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Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Doctor of Philosophy

| Year this degree conferred — Année d'obtention de ce grade | Name of Supervisor — Nom du directeur de thèse |
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# EFFECTS OF SALMON GONADOTROPHIN AND SEX STEROIDS ON PLASMA LIPIDS IN THE GOLDFISH, *CARASSIUS AURATUS*

by

© MURRAY DOUGLAS WIEGAND

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1979

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Effects of salmon gonadotrophin and sex steroids on plasma lipids in the goldfish, *Carassius auratus*," submitted by Murray Douglas Wiegand in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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## ABSTRACT

Female goldfish were held under conditions of 12°C and 12L/12D photoperiod during three phases of the sexual cycle. A pre-treatment blood sample was taken after 2 weeks of acclimation. Comparison of pre-treatment levels of plasma lipids from the three experiments revealed that the concentration of triglycerides (TG) increased and total cholesterol (TC) decreased with increasing ovarian size. Plasma lipid phosphorus (LP) levels were slightly higher in sexually mature fish than in sexually regressed fish. Two weeks after the pre-treatment blood sample, the fish were injected intraperitoneally with salmon gonadotrophin (SG-G100) or control solution for 3 days, after which a post-treatment blood sample was taken. Throughout the experiments, the goldfish were fed twice daily except for a 24 hour starvation period prior to each blood sample. In sexually regressed and maturing fish injection of SG-G100 caused increased plasma TG levels (compared to pre-treatment) in fish with small ovaries, changing to decreased levels in fish with larger ovaries. A similar effect was also seen in maturing fish with plasma TC; these effects were abolished by castration or by keeping fish at 21°C. SG-G100 had little effect on plasma LP. The results suggest that gonadotrophin causes a net mobilization of lipid in fish with small ovaries (presumably via sex steroids) and accelerated ovarian uptake of lipid in fish with larger ovaries.

Using a similar protocol to that described above, the effects of sex steroids on plasma lipids in female goldfish were studied in

sexually regressed and maturing fish at 12°C, and in post-ovulatory-regressed fish at 21°C. Intraperitoneal injection of oestrone ($E_1$), but not oestradiol ($E_2$), raised plasma TG concentrations in regressed fish, but neither oestrogen had an effect on plasma TG in maturing fish at 12°C or in post-ovulatory-regressed fish at 21°C. Progesterone injection caused high levels of plasma TG in maturing fish at 12°C. Fish injected with $E_1$ or $E_2$ had higher plasma TC levels post-treatment compared to control fish in both experiments at 12°C, but $E_1$ and $E_2$ were without effect on plasma TC at 21°C. Both $E_1$ and $E_2$ raised plasma LP levels in all three experiments. Testosterone generally had little effect on plasma lipids. These results support the hypothesis that oestrogen is involved in lipid mobilization in teleosts, and it appears that this effect is sensitive to warm temperature. There was no support for a mammalian-like, progesterone-stimulated system for clearance of plasma TG in the female goldfish.

Plasma free fatty acids (FFA) and hormonal effects thereon were found to be sensitive to the length of fasting period prior to blood sampling. Plasma FFA levels increased with length of fasting period in goldfish maintained at 12°C. A single injection of T caused elevated plasma FFA levels in fish sampled after 3 days of fasting. $E_2$ was without effect. Both hormone- and vehicle-injected fish had high levels of plasma FFA after 3 injections and 7 days of fasting, compared to uninjected fish. These results are consistent with androgen stimulating mobilization of FFA in fasted fish but the importance of such a system in fed fish is unclear.

Preliminary investigations using electrophoresis and selective

precipitation revealed two major groups of plasma lipoproteins in male goldfish. Oestrogen treatment altered the precipitation characteristics of the lighter group.

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. R.E. Peter for his enthusiastic supervision of my graduate work in the Department of Zoology. His initial suggestion of the topic and advice concerning both the research and preparation of the manuscript are appreciated. In addition, I am grateful for the independence with which I was able to pursue this study and the generous financial support. Dr. Peter also performed the hormone pellet implants in the brain described in Chapter 3.

I am grateful to Alice Hontela, Christine Paulencu and Dr. Peter for their assistance with blood sampling and histological procedures.

I wish to thank my supervisory committee, Drs. W.C. Mackay, J.R. Thompson and S.E. Zalik for their interest and helpful suggestions throughout the study. Thanks also go to Drs. Mackay and Zalik and to Brian Medford for generous loans of equipment and to those who assisted with care of the fish, especially Pat Valastin.

Alan Cook critically reviewed the manuscript and Elizabeth Cook worked diligently in its preparation.

Financial assistance was provided through a National Research Council of Canada 1967 Science Scholarship and a Graduate Teaching Assistanceship from the University of Alberta.

Finally, I wish to express sincere appreciation to my wife, Nancy, for her unwavering moral support during the entirety of my graduate studies.

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# GENERAL INTRODUCTION

Temperate zone teleost fishes are seasonally-breeding animals which undergo annual cycles of gonad growth (recrudescence) in preparation for spawning. In females, at the conclusion of the spawning season, the ovary may comprise as little as 1% of the total body weight and, in preparation for the next spawning period, the ovary can grow to as much as 20% of the body weight or more. Metabolic adaptation of these fishes to ovarian recrudescence must include provision of material for deposition in the growing ovary where large quantities of nutrient are stored in the oocytes for subsequent utilization by the developing embryos. In addition, energy must be provided to support both hypertrophy and hyperplasia of ovarian tissue. Studies on the metabolic adaptation to ovarian growth in teleosts have included both measurements of changes in tissue composition through all or part of the sexual cycle, including blood composition, and also experimental induction with hormones of specific metabolic alterations pertinent to recrudescence.

One postulate that has arisen from studies on changes in tissue composition during the sexual cycle is that lipid is important in gonad recrudescence, both as an energy source and also as a constituent deposited in the growing ovary. Depletion of stored lipid reserves has been observed to accompany ovarian growth in the sockeye salmon, *Oncorhynchus nerka* (Idler and Bitners 1958, 1959, 1960), the cod, *Gadus morhua* (Jangaard *et al.* 1967; *G. morhua callarias*, Shatunovskiy 1971), the scorpionfish, *Scorpaena porcus* (Shchepkin 1971), the haddock, *Melanogramus aegelfinus* (Shevchenko 1972), the golden shiner,

*Notemigonus crysoleucas* (de Vlaming 1975) and in several species studied by Shul'man (1974). These observations have led to the hypothesis that fat is transferred from extra-ovarian stores to the ovary for storage and oxidation. Lipid has been reported to comprise between 10% and 40% of the dry weight of teleost ovaries (Lapin 1973; Lizenko *et al.*1973; Medford and Mackay 1978).

In addition to the data on changing lipid content of various tissues in teleosts during the reproductive cycle, changes in plasma concentrations of various lipids through the sexual cycle have been described in several species. De Vlaming *et al.* (1977b) found high levels of total plasma lipids in saline-injected (control) sexually mature goldfish, *Carassius auratus*, in May, intermediate levels in fish in the early stages of gonadal recrudescence in November, and low levels in recrudescing fish in January. In the flounder, *Platichthys flesus*, there are seasonal variations in total plasma lipids with the lowest levels occurring at the end of the spawning season (May) and also in November, and peak levels in August and February (Petersen and Emmersen 1977); in these fish, plasma lipid phosphorus increases with increasing ovarian size up to a gonado-somatic index (GSI, defined as the weight of the gonad expressed as a percentage of total body weight) of 10% and declines at higher GSIs. McCartney (1967) found that serum lipid phosphorus (a measure of phospholipids) and cholesterol in brown trout were at minimal levels during the spawning season (September to November); peak levels of cholesterol were found in January and the highest levels of lipid phosphorus occurred immediately prior to the spawning season. Plasma

cholesterol levels have been found to decline as ovarian growth proceeds in migrating *O. nerka* (Idler and Tsuyuki 1958) and in the catfish, *Heteropneustes fossilis* plasma cholesterol declines between the preparatory and prespawning phases of ovarian development and increases again during the spawning season (Singh and Singh 1979). Changes in the fractional composition of plasma lipids with gonadal development have been described in *G. morhua callarias* (Shatunovskiy 1971) and *P. flesus bogdanovi* (Lapin 1973). These studies suggest an involvement of plasma lipids in the process of gonad growth in teleosts. However, since most of the foregoing observations were made on fish sampled under varying environmental conditions, effects of factors other than reproductive state, such as temperature and photoperiod cannot be excluded. Studies conducted under standarized environmental conditions are needed to clarify the relation between plasma lipid concentrations and reproductive condition.

In the area of endocrine control of gonad recrudescence in teleosts, attention has dwelt largely on hormonal induction of synthesis in the liver, appearance in the blood and uptake by the ovary of a specific phospholipoprotein, the yolk precursor vitellogenin (VG) (Aida *et al.* 1973a,b; Campbell 1978; Campbell and Idler 1976; Emmersen and Petersen 1976; Emmersen *et al.* 1979; Hori *et al.* 1979). Less attention has been paid to hormonal effects on other metabolites, including lipids, despite a presumption of the importance of lipids in the process of recrudescence. In general, injection of oestrogens has been found to increase plasma concentrations of a variety of lipids or lipoproteins in several teleosts

(Bailey 1957; Ho and Vanstone 1961; Urist and Schjeide 1961; Plack and Pritchard 1968; Takashima *et al.* 1972; Aida *et al.* 1973a; de Vlaming *et al.* 1977b; Korsgaard and Emmersen 1979). From these data, it has been hypothesized that oestrogen acts to mobilize lipids into the plasma for use by the growing ovary and inclusion in the oocytes. However, oestrogen alone cannot support all phases of ovarian growth; in particular, it does not stimulate incorporation of VG into the ovary (Upadhyay 1977; Campbell and Idler 1976; Sundararaj and Goswami 1968) whereas gonadotrophic preparations have this effect (Campbell and Idler 1976; Campbell 1978). Comprehensive studies on effects of a wider variety of hormones on lipid metabolism in relation to reproduction and studies conducted at more than one temperature or reproductive state are generally lacking.

Acclimation temperature should be an important consideration in studies of metabolic effects of reproductive hormones because of the varying influences that temperature has been found to exert on recrudescence in teleosts (for review, see Peter and Crim 1979). In the goldfish, some phases of ovarian development appear to be inhibited at temperatures over 20°C (Yamazaki 1965; Gillet *et al.* 1978).

One plasma lipid class that has largely been ignored in studies on gonad recrudescence in teleosts is free fatty acids (FFA). In mammalian systems, when triglyceride (the lipid class which comprises the bulk of labile stores) is mobilized for oxidation by other tissues, FFA are released into the plasma. In teleosts, studies of plasma FFA concentrations through the sexual cycle are lacking.

However, if plasma FFA are utilized in the process of ovarian recrudescence, it could be expected that appropriate hormonal manipulation would affect their concentrations.

Plasma lipids are transported in association with proteins, forming complexes known as lipoproteins. Isolation of plasma or serum lipoproteins and partial characterization of the lipid moieties has been performed for a number of teleost species. However, few studies of effects of sex hormones or state of sexual maturity on lipoproteins in teleosts are available.

The present study was designed to test the effects of several hormones on plasma lipid concentrations in the goldfish, the majority of the work being conducted with females. The experimental design used in the work reported in Chapters 1 and 2 allowed comparison of concentrations of three classes of plasma lipids, triglycerides (TG), total cholesterol (TC) and lipid phosphorus (LP) in fish maintained under standardized conditions at various stages of the sexual cycle. The effects of salmon gonadotrophin (SG-G100) and several sex steroids on plasma TG, TC and LP are reported in Chapters 1 and 2, respectively. The study of plasma FFA required modification of the protocol used for the other lipids; experiments on plasma FFA are reported in Chapter 3. Preliminary studies on goldfish plasma lipoproteins are reported in Chapter 4.

# Chapter 1. PLASMA TRIGLYCERIDES, CHOLESTEROL AND PHOSPHOLIPIDS DURING THE SEXUAL CYCLE IN FEMALE GOLDFISH AND THE EFFECTS OF SALMON GONADOTROPHIN.

## INTRODUCTION

Although several studies have indicated that oestrogen may effect lipid mobilization in teleosts, the possibility of a direct action of gonadotrophin on lipid metabolism, that is independent of oestrogen, has received little attention. By histological methods, Funk *et al.* (1973) demonstrated that partially purified salmon gonadotrophin (SG-G100) accelerated development of late perinucleolar and early yolk vesicle stage oocytes, with concomitant deposition of lipid in the oocytes in pink salmon, *Oncorhynchus gorbuscha*. Uphadyay (1977) showed that injection of a glycoprotein gonadotrophin caused the accumulation of "lipid bodies", but not vitellogenin, in developing oocytes in rainbow trout, *Salmo gairdneri*. These results suggest that gonadotrophin may be the stimulus for the gonad to sequester lipids. Information on effects of gonadotrophin on plasma lipids in teleosts is lacking. In the amphibian, *Xenopus laevis*, injection of mammalian follicle stimulating hormone causes lowering of plasma lipid levels (Follett and Redshaw 1968).

The work presented in this chapter was undertaken to examine the effects of a fish gonadotrophin (partially purified salmon gonadotrophin, SG-G100) on the concentrations of several plasma lipids in sexually regressed, maturing and mature female goldfish and castrate females acclimated to cold (12°C), and post-ovulatory

fish acclimated to warm (21°C) temperatures. The experiments also allowed comparison of plasma lipid concentrations in fish held under standardized conditions at three different stages of the sexual cycle.

## MATERIALS AND METHODS

### I. Source and Maintenance of Fish

Common or comet variety goldfish were purchased from Grassyforks Fisheries, Martinsville, Indiana, U.S.A. and held in 4800 litre tanks under simulated natural (Edmonton) photoperiods at 12°C - 15°C until use. At the beginning of the experimental acclimation period, the fish were weighed and tagged on the operculum with size 1 Monel tags (National Band and Tag Co., Newport, Ky., U.S.A.) for individual identification. Experiments 1.1 to 1.4 were conducted in 96 litre flow-through aquaria; Experiment 1.5 was conducted in standing water 41 litre aquaria.

### II. Experiments

*Standard Experimental Protocol*

Female goldfish were acclimated to conditions of either 12 ± 1°C or 21 ± 1°C and a 12 hours light/12 hours dark (12L/12D) photoperiod for 2 weeks. An initial (pre-treatment) blood sample was then taken. Two weeks were allowed for recovery under the same environmental conditions prior to commencement of the experimental treatment. One group of fish was injected with SG-G100, while the injected controls received bovine serum albumin (BSA) and another control group was uninjected. The fish were treated for 3 days, and on the fourth day the second (post-treatment) blood sample was taken. Throughout the experiments the fish were fed twice daily, *ad libitum*, with Ewos Salmon Grower Extra pellets, size 5P (Ewos Aquaculture International) except for a

24 hour starvation period prior to each blood sample. After the second sample, the fish were weighed, sacrificed and the ovaries were removed, weighed and fixed. Any fish that ovulated during the experiments (i.e. after SG-G100 treatment) were discarded. The ovary was processed by standard histological procedures and ovary condition scored according to Hontela and Peter (1978).

*Specific Experimental Conditions*

*Experiment 1.1*

In order to induce ovarian regression, female goldfish were held at 28°C - 30°C for 28 days in June, 1977. They were then injected once with 100 IU human chorionic gonadotrophin (HCG) (Ayerst)/fish and on the next day with 80 IU HCG/fish. After the first injection of HCG, ovulated eggs were stripped from 7 fish and, after the second injection, a few eggs were stripped from 2 fish. The fish were then considered to be regressed. The temperature was lowered to 12°C over a period of 3 days. After these manipulations, the fish weighed 26.8 g(S.D. = 5.4) and were subjected to the standard protocol at 12 ± 1°C. At the end of the experiment the mean GSI was 1.4% (S.D. = 0.8); these fish were designated as sexually "regressed".

*Experiments 1.2 and 1.3*

Female goldfish weighing 28.9 g (S.D. = 4.7) (Experiment 1.2, January 1977) and 25.2 g (S.D. = 4.6) (Experiment 1.3, May 1977) were subjected to the standard protocol at 12 ± 1°C. At the end of Experiment 1.2 the mean GSI was 5.6% (S.D. = 3.0); these fish were undergoing ovarian recrudescence and were designated as sexually "maturing". At the end of Experiment 1.3 the mean GSI was 12.9% (S.D. = 2.9); these fish were designated as sexually "mature".

*Experiment 1.4*

Female goldfish which had previously ovulated were kept at 21 ± 1°C in May 1978, and injected with 100 IU HCG (Sigma)/fish on 2 consecutive days. The temperature was raised to 28°C - 30°C over a period of 2 days and the fish were held at that temperature for 10 days. The temperature was returned to 21 ± 1°C and 3 more daily injections of HCG (100 IU/fish) were given. A few ovulated eggs were stripped from 2 fish after the last injection. The fish were then considered to be "post-ovulatory-regressed". Experiment 1.4 was carried out using the standard protocol at 21 ± 1°C. The weight of the fish at the commencement of acclimation to 21 ± 1°C and 12L/12D photoperiod was 34.3 g (S.D. = 8.2). At the end of the experiment the mean GSI was 2.1% (S.D. = 1.8).

*Experiment 1.5*

Prior to ovariectomy, female goldfish were held at 21 ± 1°C on a simulated natural (Edmonton) photoperiod in April 1978, and injected on 2 consecutive afternoons with 100 IU HCG (Sigma)/fish after which the temperature was raised to 28°C - 30°C over a 2 day period. After 6 days at 28°C - 30°C, the temperature was lowered to 15°C over a 3 day period. This HCG/temperature regime effected partial regression of the ovaries. After 2 days at 15°C, the fish were transferred to 41 litre standing water aquaria at 12 ± 1°C for the experiment (3 or 4 fish per aquarium). The water in each aquarium was filtered through a 2 litre glass wool and charcoal bed filter external to the tank. The fish were held in dechlorinated tap water to which the following salts were added, to aid in healing from the operation: NaCl, 3.93 g/l; KCl, 0.17 g/l; $MgSO_4$, 0.19 g/l; $KH_2PO_4$, 0.21 g/l; $CaCl_2 \cdot 2H_2O$,

0.25 g/l and $NaHCO_3$, 1.40 g/l. One half of the water in each aquarium was changed about every three days, and in all tanks at two days prior to each blood sampling. Salts were replenished when the water was changed.

After being held under these conditions for 3 days, twenty-nine fish were ovariectomized over a period of 4 days. For the operation, the fish were anaesthetised with tricaine methanesulphonate (TMS) and a wet tissue was wrapped around the head and operculum. The second row of scales below the lateral line on the left side was removed, starting with the second scale behind the operculum and terminating two scales behind the vent. An incision along this line was then made with a scalpel and the opening of the flank was completed with scissors. The entire ovary could then be exposed and removed along with most of the oviduct. The wound was sutured with between 12 and 15 stitches of polyester thread and the operation was then repeated on the right side. Stitches were removed starting from day 4 post-operatively. Food, Tetra-Min (TetraWerke, Melle, W. Germany) mixed with Terramycin (Pfizer Co. Ltd., Montreal), was first offered on Day 6 postoperatively and by Day 12 all fish had been observed to feed. An acclimation period of 2 weeks at 12 ± 1°C and 12L/12D photoperiod was begun at 12 days postoperatively. From that point, the fish were handled according to the standard protocol except that they were held in salt water (described above) and fed once per day on Tetra-Min mixed with Terramycin. Twenty-one of the fish survived the length of the experiment. At the time of castration, the mean weight and GSI were 31.2 g (S.D. = 5.5) and 9.4% (S.D. = 3.7), respectively.

III. Solutions and Injections

Salmon gonadotrophin (SG-G100) was purchased from the Fisheries and Marine Service, Environment Canada, Vancouver Laboratory, West Vancouver, B.C. and was selected for the experiments because of its demonstrated ability to support vitellogenesis and other reproductive functions in goldfish (Yamazaki and Donaldson 1968a,b, 1969). BSA was purchased from Sigma. The proteins were dissolved in double distilled deionized water immediately prior to use. For Experiments 1.1, 1.2, 1.3 and 1.5, the concentration of the protein solutions was 200 mg/ml and the injected dosage was 2.0 µg/2g body weight of the fish. For Experiment 1.4 the concentration of the protein solutions was 500 µg/ml and the injected dosage was 5.0 µg/2g. For injection, the fish were removed from the aquaria into buckets containing water at the experimental temperature. Injections were given intraperitoneally, without anaesthesia, using a 27 gauge needle on a 1 cc disposable syringe at 15:30 - 17:00 hr. (lights on in the experimental photoperiod at 08:00). The afternoon feeding was approximately 30 minutes after completion of injections, except on the day of the last injection when no food was offered in the afternoon.

IV. Blood Sampling Procedure

Fish were removed from the aquaria between 09:30 - 11:00 and anaesthetized in TMS (0.03% w/v). Blood (approx. 0.45 ml) was drawn from the caudal vasculature through a 23 gauge needle into a 1 cc disposable syringe containing approximately 4 mg ethylenediamine tetra-acetic acid, disodium salt. Each fish was returned to its aquarium

after bleeding. The blood was expelled into polystyrene tubes and plasma was separated by centrifuging at 4°C. One plasma aliquot, usually 100 µl, was used for isolation of neutral lipids (see below) and the remainder was stored at -27°C. A record of bleeding time was kept at the first sampling, and at the second sampling the fish were bled within 20 minutes of the same time of day.

V. Analytical Procedures

Neutral lipids were extracted from the plasma using Lipo-Frax® columns and adsorbent manufactured by Analytical Products Inc., Belmont, Ca., U.S.A. Fresh plasma (usually 100 µl) was mixed with 5.0 ml isopropanol and the mixture was poured onto the column; neutral lipids were collected in the eluent. Triglycerides (TG) were measured by a modification of the method of Moses *et al.* (1975). Solutions were prepared according to Moses *et al.* (1975) and the following volumes were used: eluent, 0.2 ml; sodium methoxide solution, 0.1 ml; sodium periodate solution, 0.1 ml; ammonium acetate-acetoacetone solution, 0.5 ml. The incubations were carried out according to Moses *et al.* (1975) and absorbance of the final product was measured at 410 nm in a Unicam SP1800 dual beam spectrophotometer. Total cholesterol (TC) was measured by a modification of the method of Chiamori and Henry (1959). Eluent from the Lipo-Frax column (0.5 ml) was dried under nitrogen at 60°C and redissolved in 2.5 ml of 0.03% anhydrous ferric chloride in glacial acetic acid. Colour was developed with the addition of 1.5 ml of concentrated sulphuric acid. After cooling, the absorbance of the product was measured at 564 nm. Standards were prepared by mixing 100 µl of stock isopropanol

solutions of trioctanoin (Eastman) or cholesterol (Sigma), 100 µl of saline and 4.9 ml of isopropanol and passing the mixture through Lipo-Frax columns. The general suitability of the Lipo-Frax system and similar assays has been reviewed ( Moses *et al.* 1975). Plasma phospholipids were estimated by measuring lipid phosphorus (LP) using a modification of the method of Baginski *et al.* (1972). Solutions were prepared according to those authors and used in the following amounts: plasma, 10 µl; ethanol-ether, 2.0 ml; nitric acid-calcium nitrate, 2.0 ml; ascorbic acid-trichloroacetic acid, 2.0 ml; ammonium molybdate, 1.0 ml and arsenite-citrate, 2.0 ml. It was found that reproducibility was enhanced by prolonging the extraction time to 30 minutes with the use of a mechanical shaker and by allowing for a room temperature incubation of 22 - 25 minutes between the addition of ammonium molybdate and arsenite-citrate. The absorbance of the product was measured at 700 nm and compared to a set of standards. All assays were performed in duplicate and the samples from the first and second bleeding of each fish were normally assayed in the same batch.

## VI. Statistical Analysis

Significance of regression coefficients compared to zero, and comparisons between regression coefficients, were by $t$ test (Steel and Torrie 1960; Sokal and Rolf 1969). Other tests used were paired and unpaired $t$ tests, one way analysis of variance followed by Duncan's New Multiple Range test, and calculation of Pearson's correlation coefficient and tests of its significance (Steel and Torrie 1960; Sokal and Rolf 1969).

VII. Glassware

Digestion tubes used in the LP assay were cleaned with boiling nitric acid and rinsed with distilled water (Baginski *et al.* 1972). All other glassware was cleaned in a sulphuric acid-sodium dichromate bath followed by repeated washings with distilled water.

# RESULTS

## I. Pre-treatment Plasma Lipid Concentrations

In Experiments 1.1, 1.2 and 1.3 analysis of plasma TG from the pre-treatment (initial) sample (taken after 2 weeks of acclimation to 12 ± 1°C and 12L/12D photoperiod and before hormone treatment) revealed that the levels were significantly dependent on the GSI of the fish; with increasing ovarian size, plasma TG increased (Fig. 1). Plasma TC in the pre-treatment samples from Experiments 1.1, 1.2 and 1.3 showed a significant inverse dependence on GSI; TC levels decreased with increasing GSI (Fig. 2). Plasma LP levels were measured in Experiments 1.1 and 1.3 but not in Experiment 1.2. The mean level in the pre-treatment sample from Experiment 1.3 (sexually mature females) was 39.5 ± 0.8 (SEM) mg/dl and in Experiment 1.1 (sexually regressed females) it was 35.9 ± 0.9 mg/dl. These means were different at the $p < 0.01$ level (Student's $t$ test). If LP is plotted against GSI as in Figs. 1 and 2, the y intercept is 35.5 mg LP/dl, the regression coefficient is 0.307 and the standard error is 0.097 ($p < 0.01$).

Plasma lipid concentrations in the pre-treatment samples from each fish were also examined for significant correlations between concentrations of pairs of lipids (Table 1). In sexually regressed, mature and post-ovulatory-regressed fish, there was a significant positive correlation between the pre-treatment levels of TC and LP. Pre-treatment concentrations of TG and LP were also positively correlated in regressed and post-ovulatory-regressed fish, but not in mature fish. Pre-treatment plasma TG and TC were significantly correlated in regressed and maturing fish but not in mature, post-

Figure 1. Regression of pre-treatment concentrations of plasma triglycerides on gonadosomatic index in sexually regressed goldfish (Experiment 1.1) (▲), sexually maturing goldfish (Experiment 1.2) (★), and sexually mature goldfish (Experiment 1.3) (■).
TG = y = plasma trigiycerides; GSI = x = gonadosomatic index; SE = standard error of the regression coefficient (slope); p = significance of difference of regression coefficient from zero; N = number of goldfish.

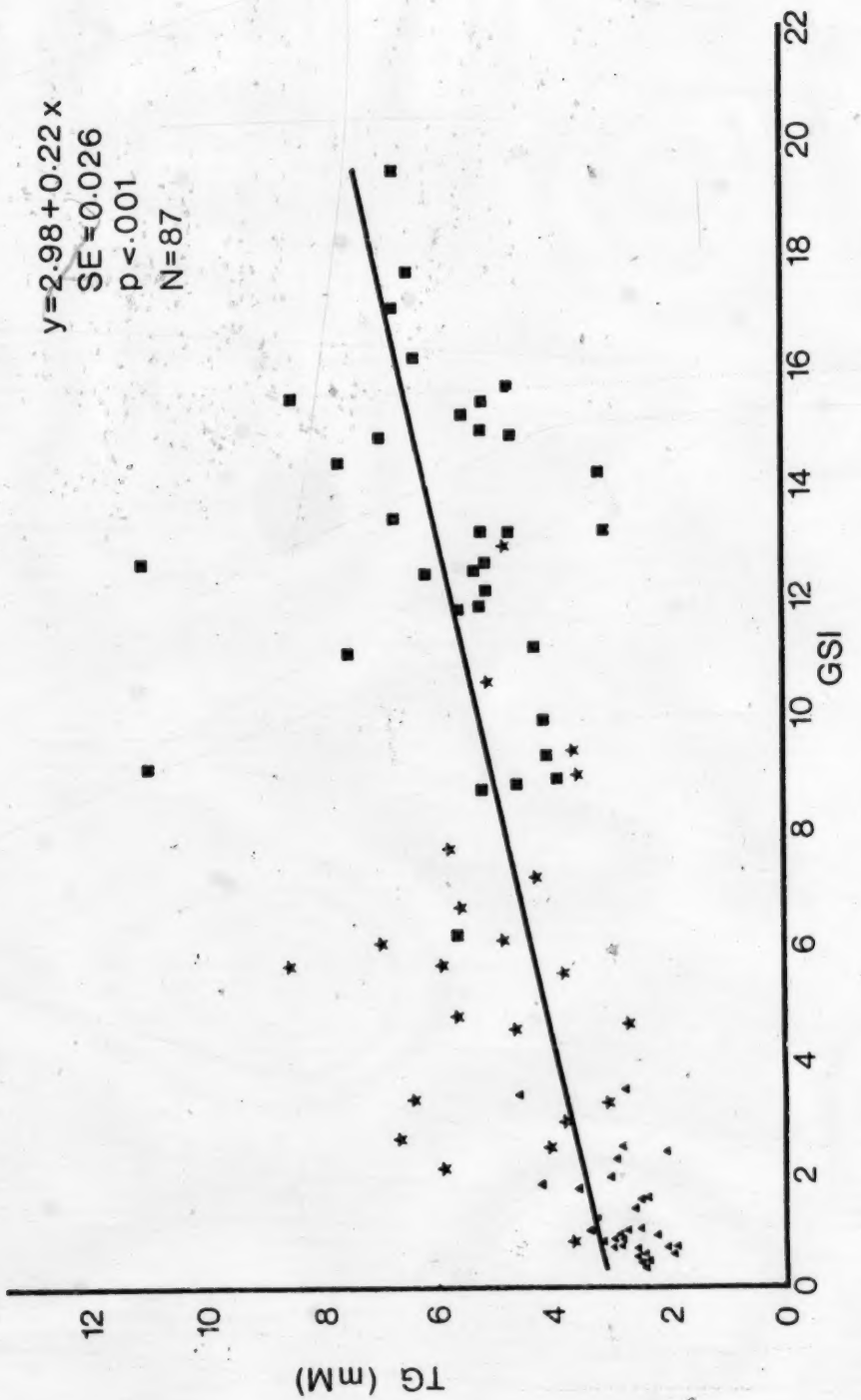

y=2·98+0·22 x
SE=0·026
p<.001
N=87
TG (mM)
GSI
0
2
4
6
8
9
10
12
0
2
4
6
8
10
12
14
16
18
20
22

Figure 2. Regression of pre-treatment concentrations of plasma total cholesterol (TC) on GSI of sexually regressed goldfish (Experiment 1.1) (▲), sexually maturing goldfish (Experiment 1.2) (★), and sexually mature goldfish (Experiment 1.3) (■).

y = Total Chol. = plasma total cholesterol. Other symbols as in Figure 1.

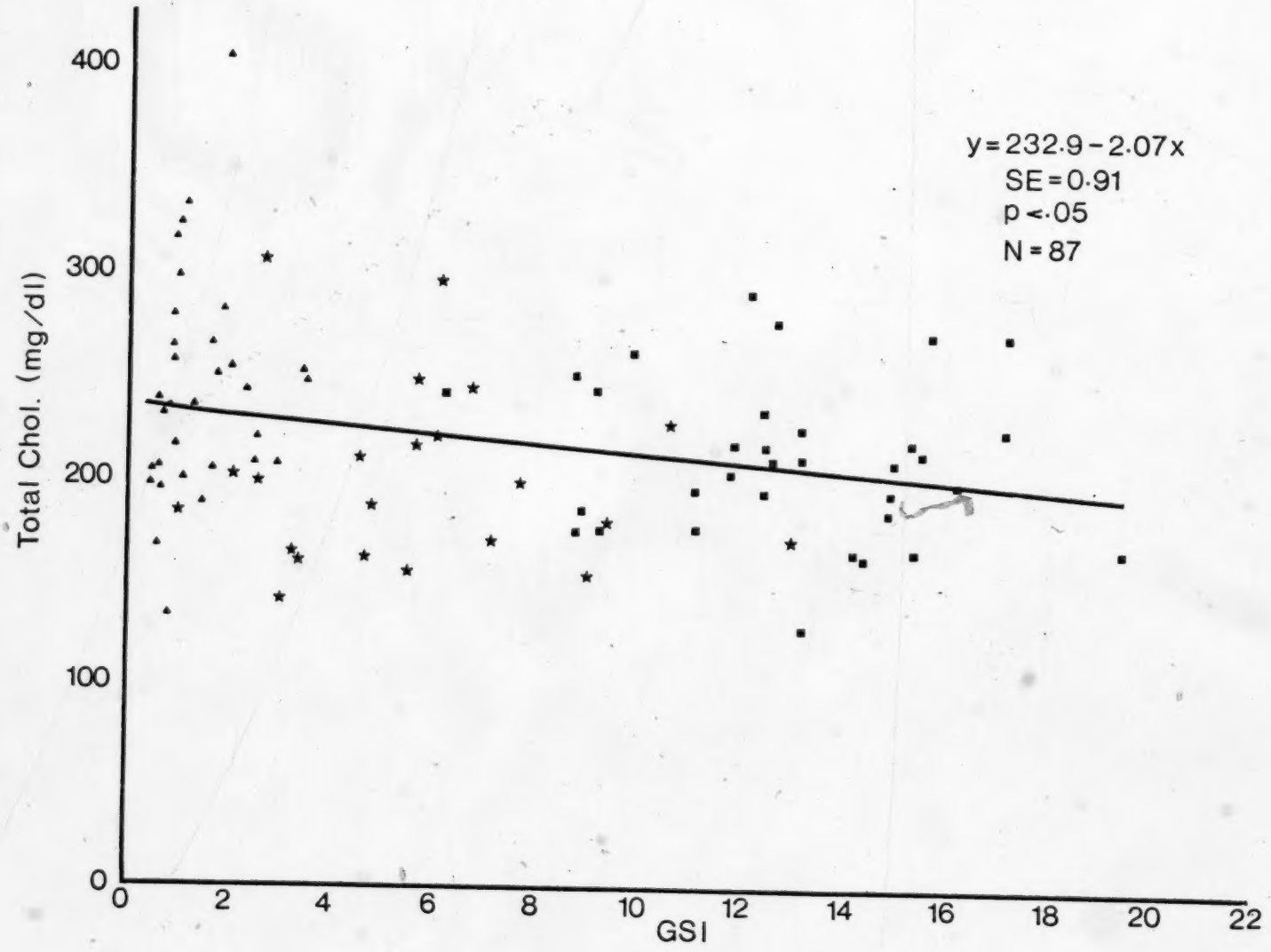

y = 232·9 − 2·07x
SE = 0·91
p < ·05
N = 87
Total Chol. (mg/dl)
400
300
200
100
0
0
2
4
6
8
10
12
14
16
18
20
22
GSI

TABLE 1. Correlation coefficients ($r$) for pre-treatment plasma concentrations of pairs of lipids in Experiments 1.1, 1.2, 1.3, 1.4 and 1.5.

| Experiment | TC vs. LP | TG vs. LP | TC vs. TG |
|---|---|---|---|
| 1.1 (Sexually regressed) | $r$ = .718<br>N = 28<br>$p$ < 0.001 | $r$ = .653<br>N = 28<br>$p$ < 0.001 | $r$ = .444<br>N = 28<br>$p$ < 0.02 |
| 1.2 (Sexually maturing) | - | - | $r$ = .519<br>N = 22<br>$p$ < 0.02 |
| 1.3 (Sexually mature) | $r$ = .441<br>N = 31<br>$p$ < 0.02 | $r$ = .297<br>N = 31<br>n.s. | $r$ = .108<br>N = 31<br>n.s. |
| 1.4 (Post-ovulatory-regressed) | $r$ = .445<br>N = 30<br>$p$ < 0.02 | $r$ = .580<br>N = 30<br>$p$ < 0.001 | $r$ = .257<br>N = 30<br>n.s. |
| 1.5 (Castrate females) | - | - | $r$ = .422<br>N = 21<br>n.s. |

NOTE: $p$ = significance of regression coefficient;

n.s. = not significant.

ovulatory-regressed or castrate fish.

## II. Effects of Salmon Gonadotrophin (SG-G100)

### *Plasma Triglycerides*

The data on plasma TG from Experiments 1.1 and 1.2 are pooled for clarity of presentation. In both sexually regressed (Experiment 1.1) and sexually maturing (Experiment 1.2) fish at 12 ± 1°C, comparison of post-treatment with pre-treatment plasma TG concentrations revealed that SG-G100 had an effect on plasma TG that was dependent on the GSI in the form of equation 1:

$$y = a - bx \qquad (1)$$

where "y" is the change in plasma TG in the individual fish between samplings (post-treatment level minus pre-treatment level), "x" is the GSI, "-b" is the slope of the line (regression coefficient) and "a" is the "y" intercept (Fig. 3). The results indicate that plasma TG levels increased in fish with small ovaries and decreased in those with larger ovaries following treatment with SG-G100. On the other hand, injection of BSA had no effect (Fig. 4). In the uninjected controls, plasma TG changed between samplings in a manner that was similar to the fish injected with SG-G100 (Fig. 5). However, the magnitude of the regression coefficient in Fig. 5 is significantly less than that in Fig. 3 ($p < 0.05$). If Experiments 1.1 and 1.2 are considered separately, the change of plasma TG between samplings in the SG-G100-treated groups still remains significantly dependent on GSI. However, there is no significant dependence of change in plasma TG on GSI in the uninjected or the BSA-injected control groups in either Experiment

Figure 3. Regression of changes in plasma TG between samplings on GSI in sexually regressed goldfish (Experiment 1.1) (★) and sexually maturing goldfish (Experiment 1.2) (◆) injected with partially purified salmon gonadotrophin (SG-G100).

△TG = change in plasma TG between samplings (post-treatment level minus pre-treatment level).

Other symbols as in Figure 1.

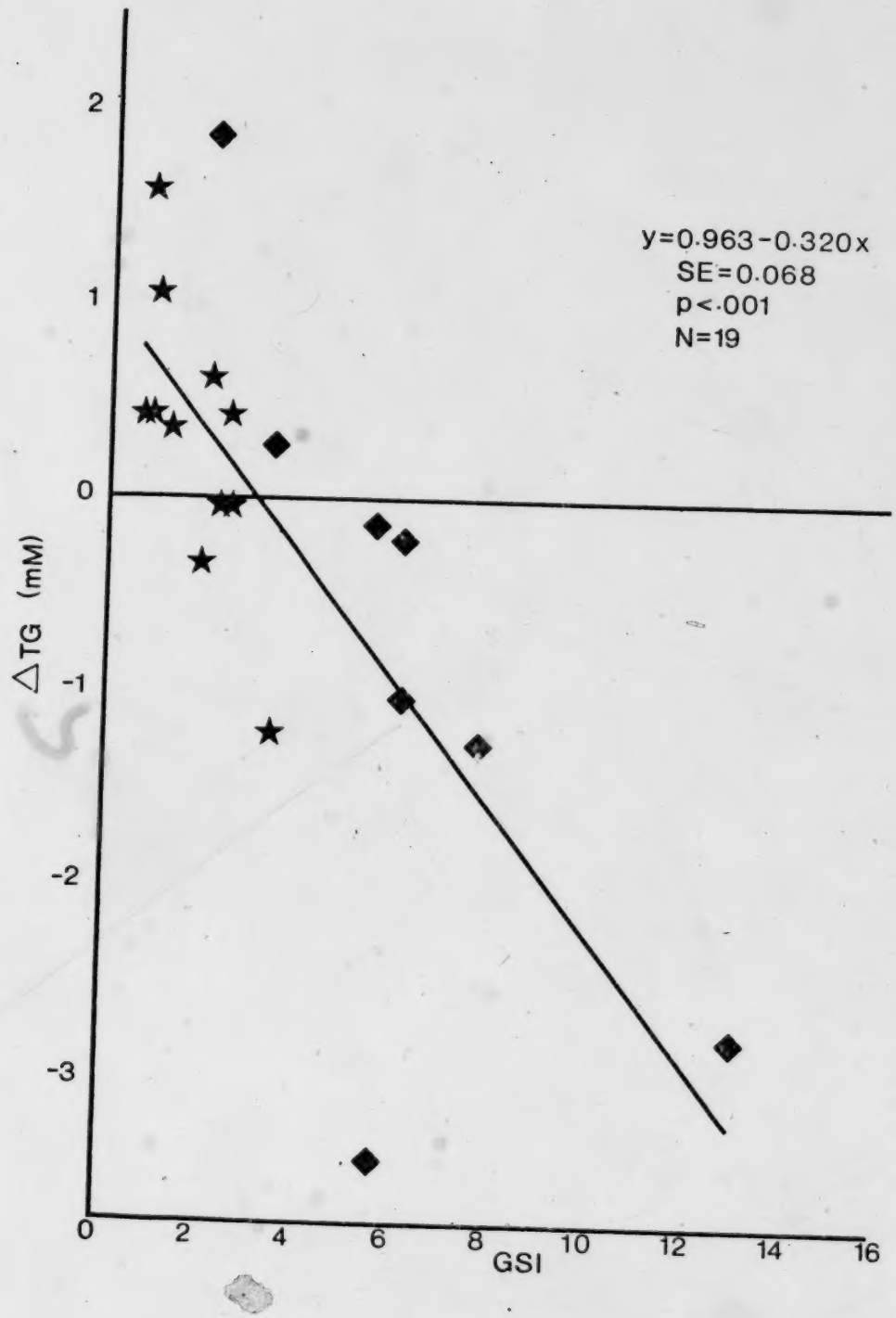

y=0.963-0.320x
SE=0.068
p<.001
N=19
ΔTG (mM)
GSI
2
1
0
-1
-2
-3
0
2
4
6
8
10
12
14
16

Figure 4. Changes in plasma TG between samplings versus GSI in sexually regressed goldfish (Experiment 1.1) (★) and sexually maturing goldfish (Experiment 1.2) (◆) injected with bovine serum albumin (BSA). There was no significant dependence of changes in plasma TG on GSI.

Symbols as in Figure 3.

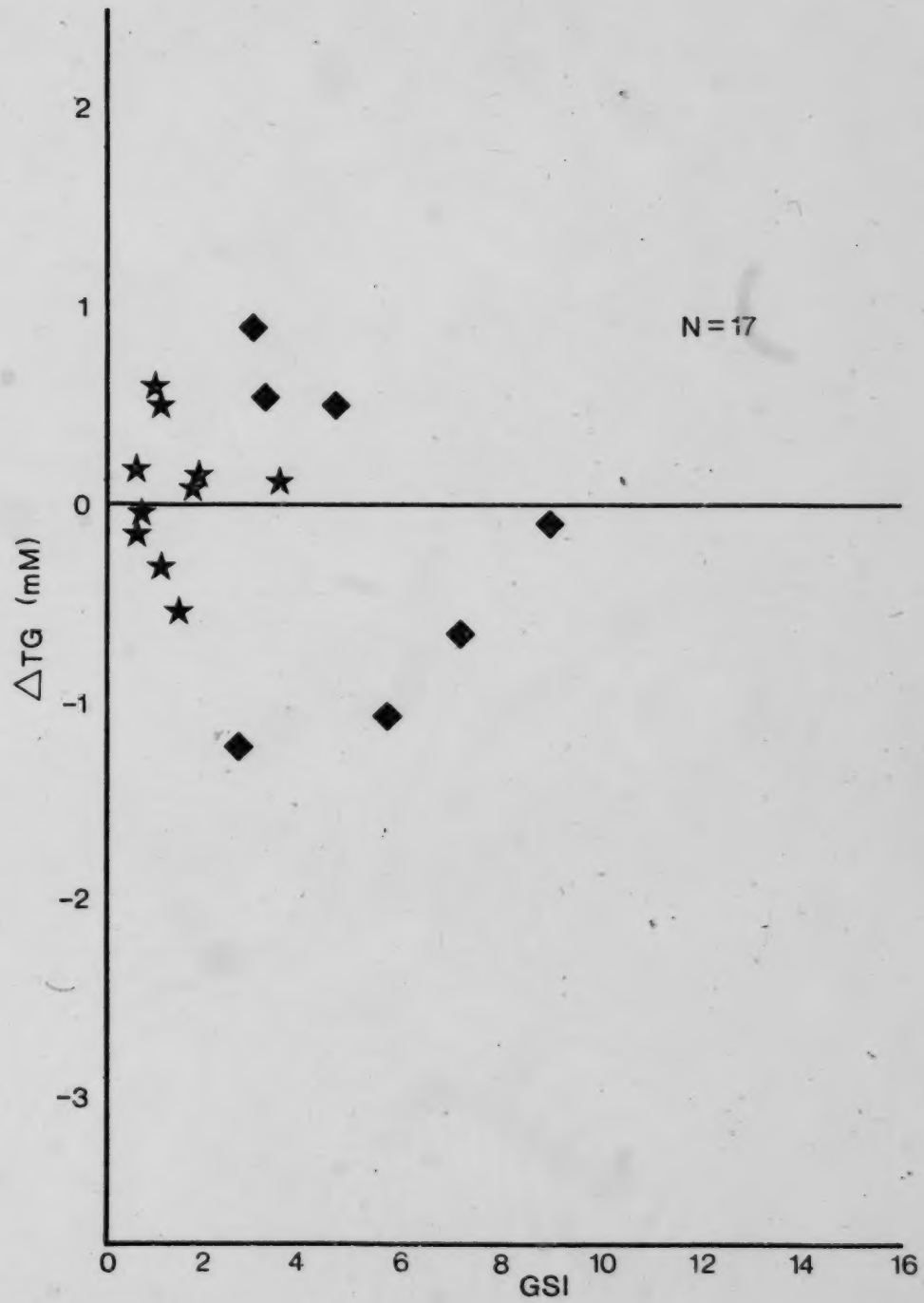
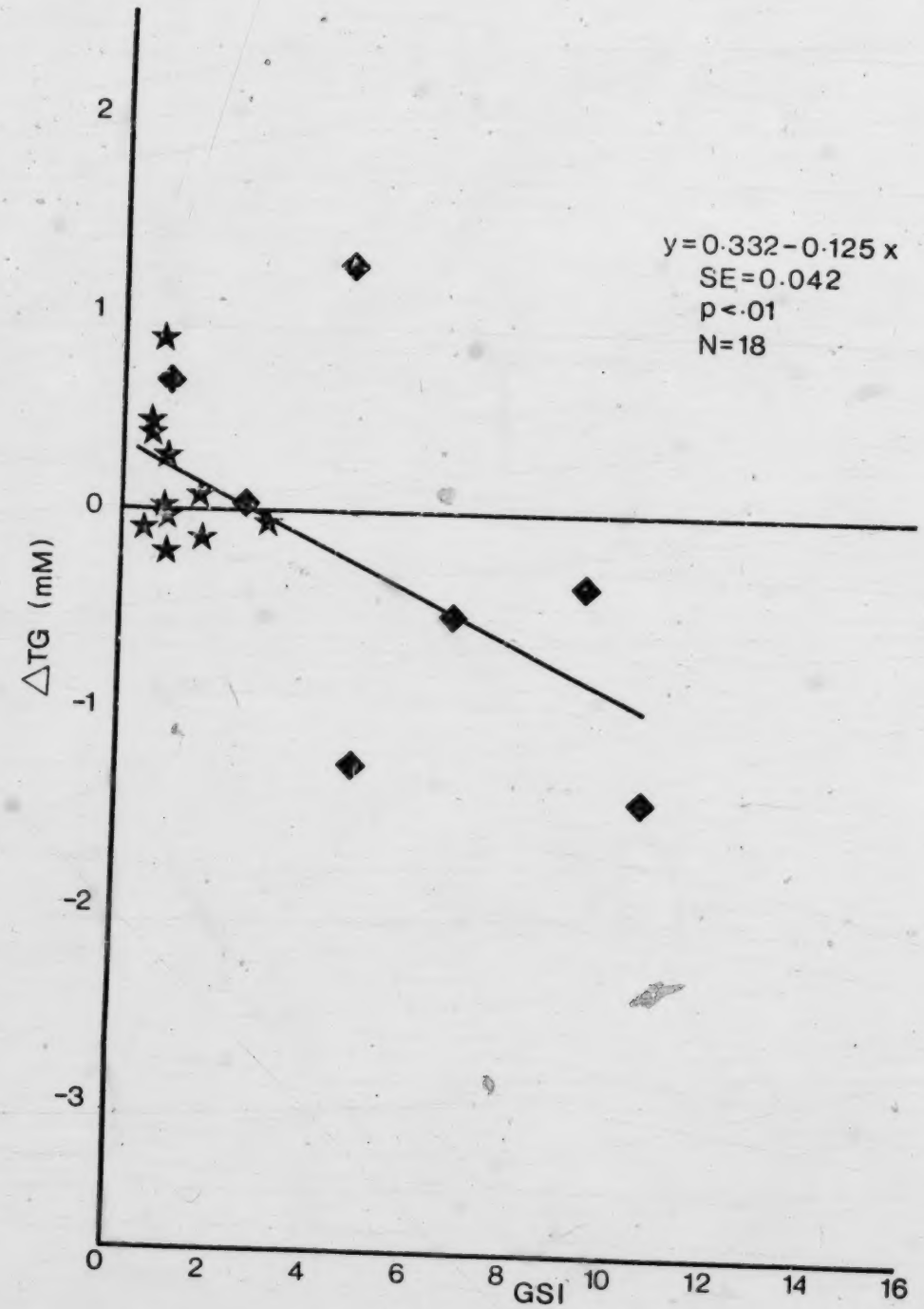

N = 17
2
1
0
-1
-2
-3
ΔTG (mM)
0
2
4
6
8
10
12
14
16
GSI

Figure 5. Regression of changes in plasma TG between samplings on GSI in sexually regressed goldfish (Experiment 1.1) (★) and sexually maturing goldfish (Experiment 1.2) (◆) that were uninjected.

Symbols as Figure 3.


y=0·332-0·125 x
SE=0·042
p<·01
N=18
2
1
0
-1
-2
-3
ΔTG (mM)
0
2
4
6
8
GSI
10
12
14
16

1.1 or Experiment 1.2 when considered alone.

In addition, changes in plasma TG in the fish that received SG-G100 in Experiments 1.1 and 1.2 were significantly correlated with a reduction in the number of perinucleolar oocytes visible in a microscope field of 1.54 $mm^2$ ($r = .479$, $p < 0.05$) and the appearance of secondary and tertiary yolk stage oocytes ($r = -.604$, $p < 0.01$). These data confirm the relationship between changes in plasma TG and GSI.

In sexually mature fish at 12 ± 1°C (Experiment 1.3), plasma TG decreased significantly in the two control groups between samplings, but not in the SG-G100-treated fish (Fig. 6). In post-ovulatory-regressed fish held at 21 ± 1°C (Experiment 1.4), control or SG-G100 treatment had no effect on plasma TG (Fig. 7). That SG-G100 did have biological effects in the mature fish and post-ovulatory-regressed fish was demonstrated by the ovulation of three fish in each experiment after SG-G100 treatment. In mature fish at 12 ± 1°C (Experiment 1.3) and in post-ovulatory-regressed fish at 21 ± 1°C (Experiment 1.4), there was no dependence of change in plasma TG between samplings on GSI. Also, in none of the experiments was there a difference between groups in the mean pre-treatment plasma TG concentrations or between groups in the mean post-treatment plasma TG concentrations (Duncan's test).

*Plasma Total Cholesterol*

Injection of SG-G100 had no effect on plasma mean TC levels in sexually mature fish at 12 ± 1°C, or in post-ovulatory-regressed fish at 21 ± 1°C (Table 2). In Experiment 1.1, on sexually regressed fish, plasma TC decreased significantly between samplings in both control

Figure 6. Plasma TG concentrations in pre-treatment and post-treatment samples from sexually mature goldfish (Experiment 1.3). Bars represent mean ± SE for the pre-treatment, or initial (i) sample and post-treatment, or second (s) sample from each group. Numbers of fish (N) and results of paired *t* test (if significant) appear above each pair of bars. There were no significant differences between groups in mean plasma TG concentrations in either the pre-treatment or post-treatment samples (Duncan's test). Uninj. Cont. = Uninjected control goldfish; Inj. Cont. = Control goldfish injected with BSA; SG-G100 = goldfish injected with SG-G100. Other symbols as in previous figures.

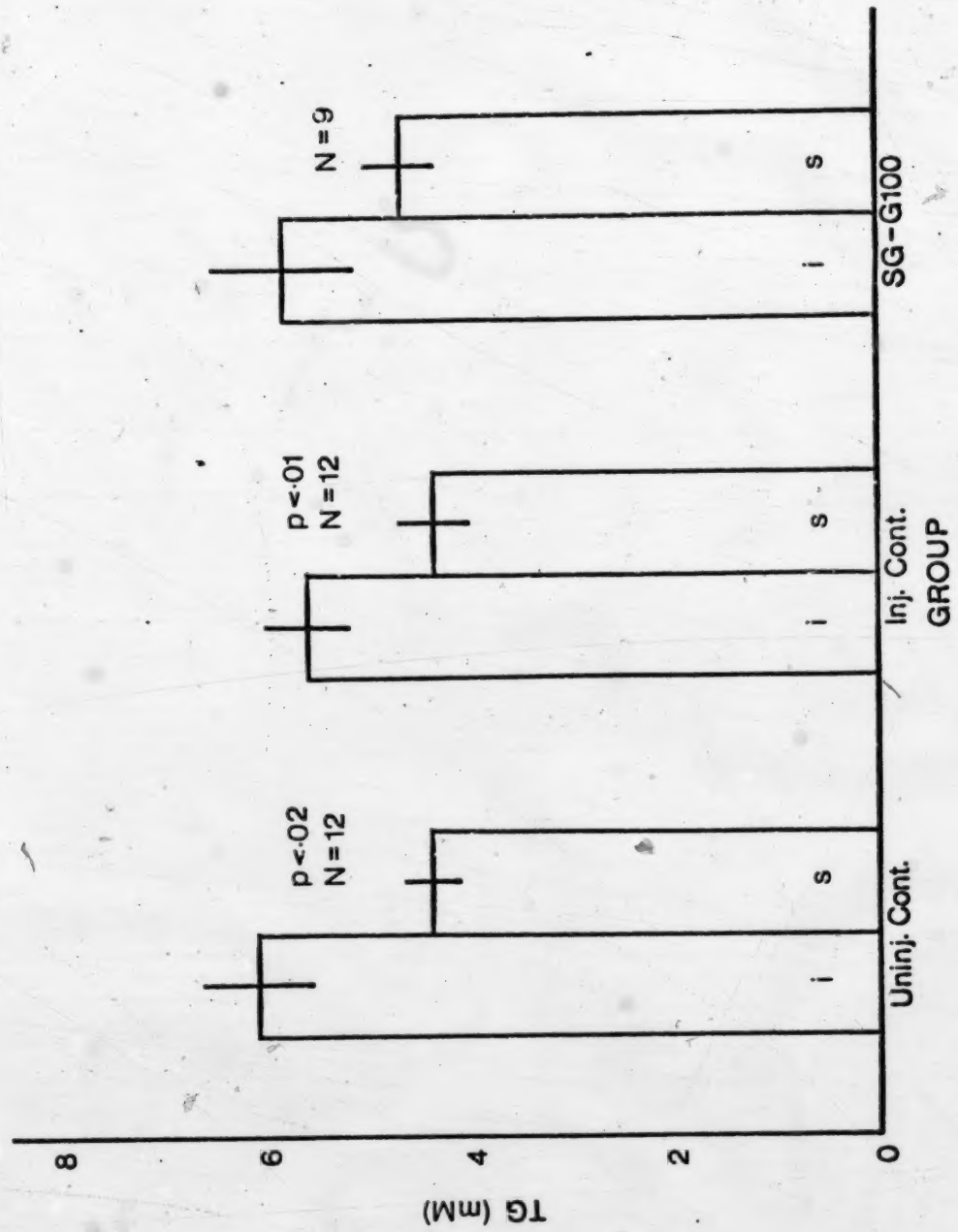

TG (mM)
0
2
4
6
8
Uninj. Cont.
Inj. Cont.
SG-G100
GROUP
p<.02
N=12
p<.01
N=12
N=9
i
s

Figure 7. Plasma TG concentrations in pre-treatment and post-treatment samples from post-ovulatory-regressed goldfish (Experiment 1.4). There were no significant differences between groups in mean plasma TG concentrations in either the pre-treatment or post-treatment samples (Duncan's test).

Symbols as in Figure 6.

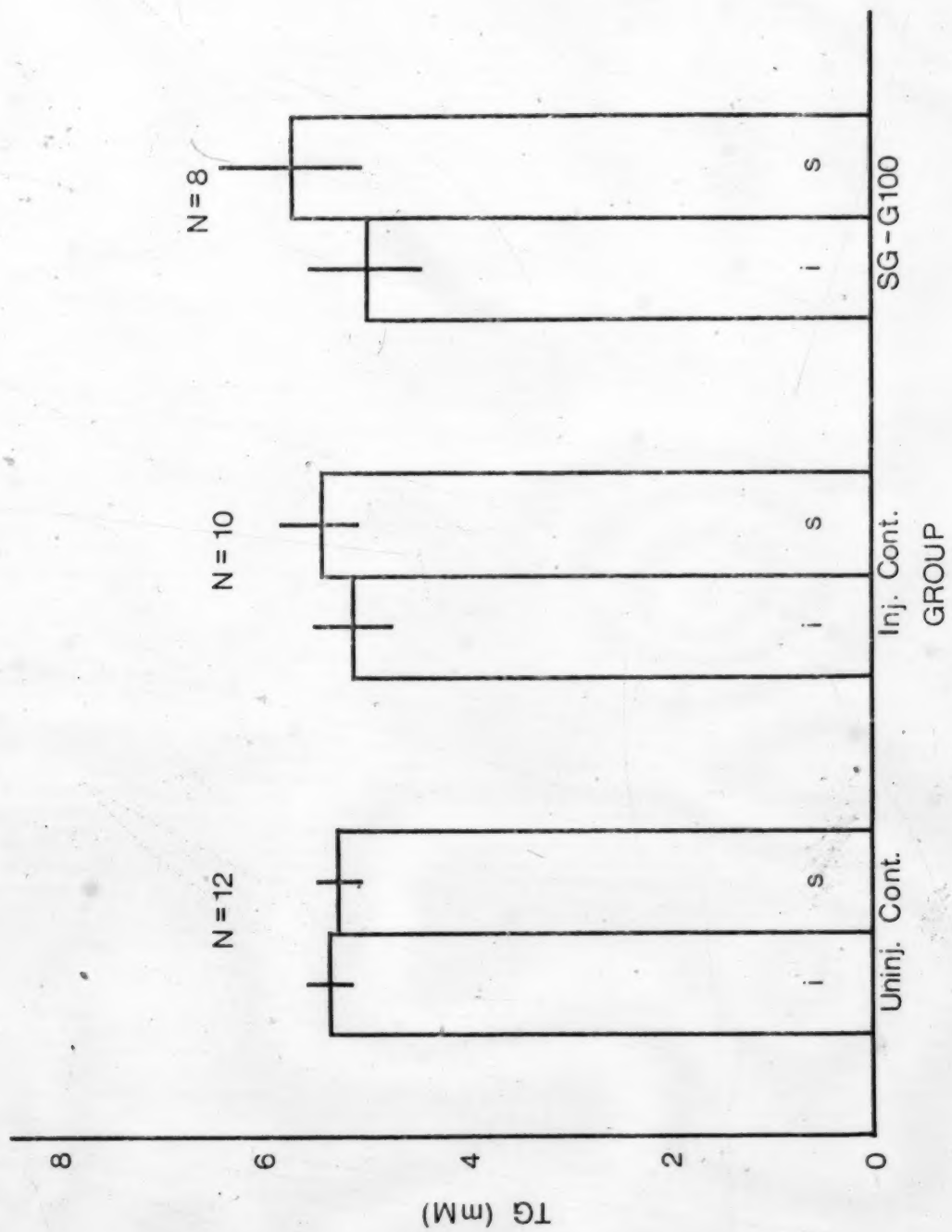

TG (mM)
0
2
4
6
8
N=12
N=10
N=8
i
s
Uninj. Cont.
Inj. Cont.
SG-G100
GROUP

TABLE 2. Concentrations of plasma total cholesterol, pre-treatment and post-treatment, in Experiments 1.1, 1.3 and 1.4

| Experiment | Plasma total cholesterol (mg/dl) | | | | | |
|---|---|---|---|---|---|---|
| | Treatment Group | | | | | |
| | Uninjected Control | | Injected Control (BSA) | | SG-G100 | |
| | Pre-treatment | Post-treatment | Pre-treatment | Post-treatment | Pre-treatment | Post-treatment |
| 1.1 (Sexually regressed) | 230.6±13.1 | 185.1±11.6 | 244.8±16.5 | 220.6±16.9 | 248.2±19.9 | 223.9±12.5 |
| | N=11 | | N=10 | | N=11 | |
| | $p < .001$ | | $p < .02$ | | n.s. | |
| 1.3 (Sexually mature) | 205.5±14.1 | 192.5±10.0 | 209.6±10.4 | 200.2±12.1 | 216.5±8.7 | 203.7±6.6 |
| | N=12 | | N=12 | | N=9 | |
| | n.s. | | n.s. | | n.s. | |
| 1.4 (Post-ovulatory-regressed) | 252.8±8.5 | 272.0±8.7 | 256.3±12.6 | 288.5±14.0 | 228.5±9.6 | 293.4±13.9 |
| | N=12 | | N=10 | | N=8 | |
| | $p < .01$ | | $p < .02$ | | $p < .001$ | |

NOTE: Mean ± S.E., numbers of fish and significance of change between samplings, evaluated by paired "$t$" test appear for each group.

n.s. = not significant.

In these experiments there was no significant difference between groups in the mean pre-treatment plasma TC concentrations or between groups in the mean post-treatment plasma TC concentrations (Duncan's test).

groups, but not in the SG-G100-treated group (Table 2). In the maturing fish only, the injection of SG-G100 had an effect on plasma TC that was dependent on GSI in the form of equation 1, similar to that discussed above for plasma TG (Fig. 8). Injection of BSA had no effect on plasma TC in this experiment (Fig. 9). Uninjected fish showed changes in plasma TC that were similar to those in the SG-G100-treated fish (Fig. 10) but the magnitude of the regression coefficient in Fig. 10 is significantly less than that in Fig. 8 ($p < 0.05$).

*Plasma Lipid Phosphorus*

In sexually regressed fish at 12 ± 1°C (Experiment 1.1), SG-G100 had no effect on plasma LP, although the BSA-injected controls showed an increase between samplings (Table 3). In post-ovulatory-regressed fish at 21 ± 1°C (Experiment 1.4), both the SG-G100- and BSA-injected groups had higher plasma LP levels in the post-treatment sample, and in mature fish at 12 ± 1°C (Experiment 1.3) there was a slight decrease in plasma LP between samplings in the SG-G100-treated fish (Table 3). In no experiment was there any significant difference between groups in the mean pre-treatment plasma LP concentrations or between groups in the mean post-treatment plasma LP concentrations (Duncan's test), and in no case were changes in plasma LP between samplings dependent on the GSI.

*Castrate Fish*

In castrate fish at 12 ± 1°C (Experiment 1.5), SG-G100 had no effect on plasma TG or TC (Figs. 11 and 12). However, plasma TC (Fig. 12) in both the pre-treatment and post-treatment samples from each group of castrate fish was lower than in the samples from the unoperated controls.

Figure 8. Regression of changes in plasma TC between samplings on GSI in sexually maturing goldfish (Experiment 1.2) injected with SG-G100.

$\triangle$ Total Chol. = change in plasma TC between samplings (post-treatment level minus pre-treatment level).

Other symbols as in Figure 3.

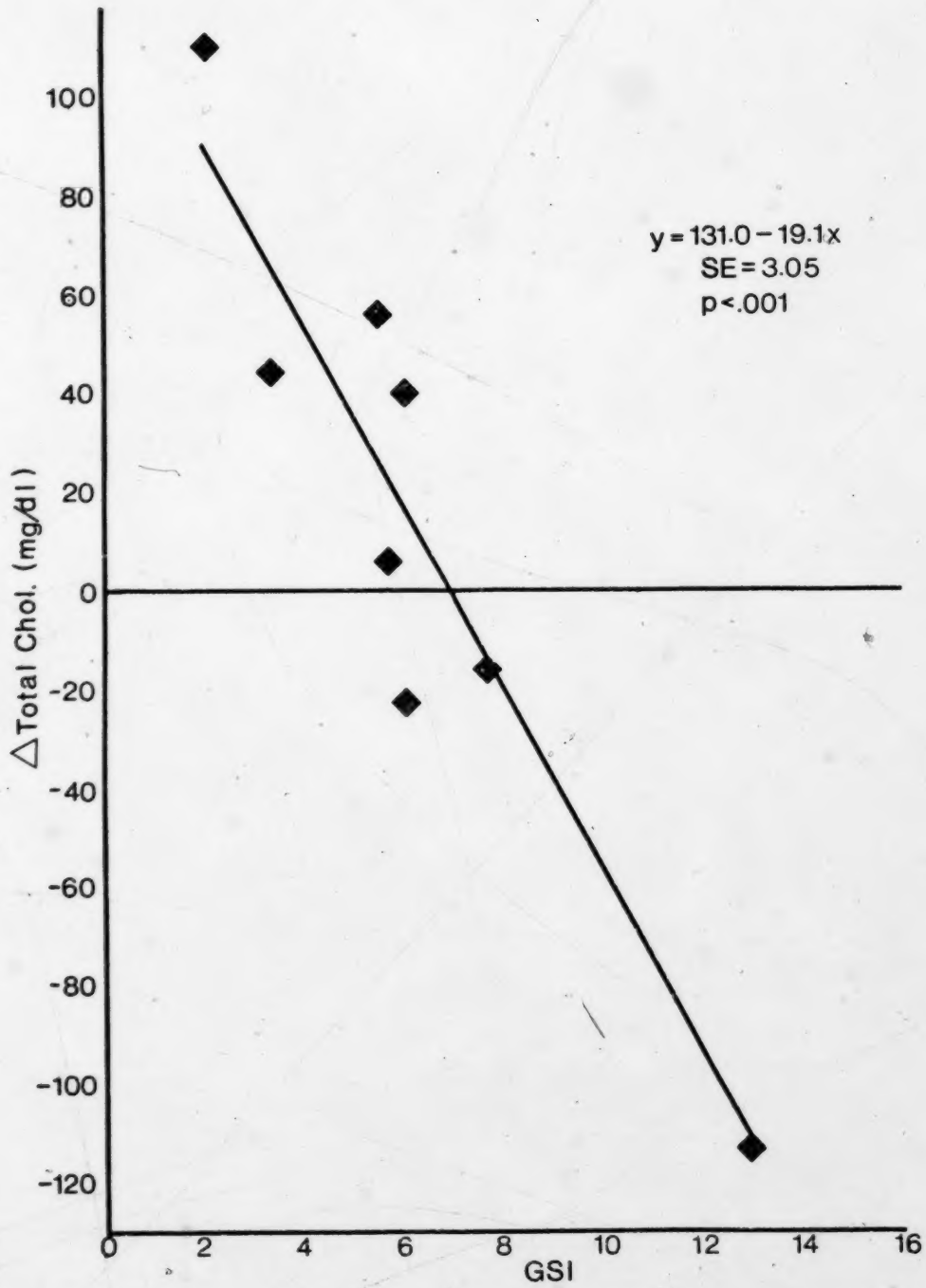

y = 131.0 – 19.1x
SE = 3.05
p<.001
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
△Total Chol. (mg/dl)
0
2
4
6
8
10
12
14
16
GSI

Figure 9. Changes in plasma TC between samplings versus GSI in sexually maturing goldfish (Experiment 1.2) injected with BSA. There was no significant dependence of changes in plasma TC on GSI. Symbols as in Figure 8.

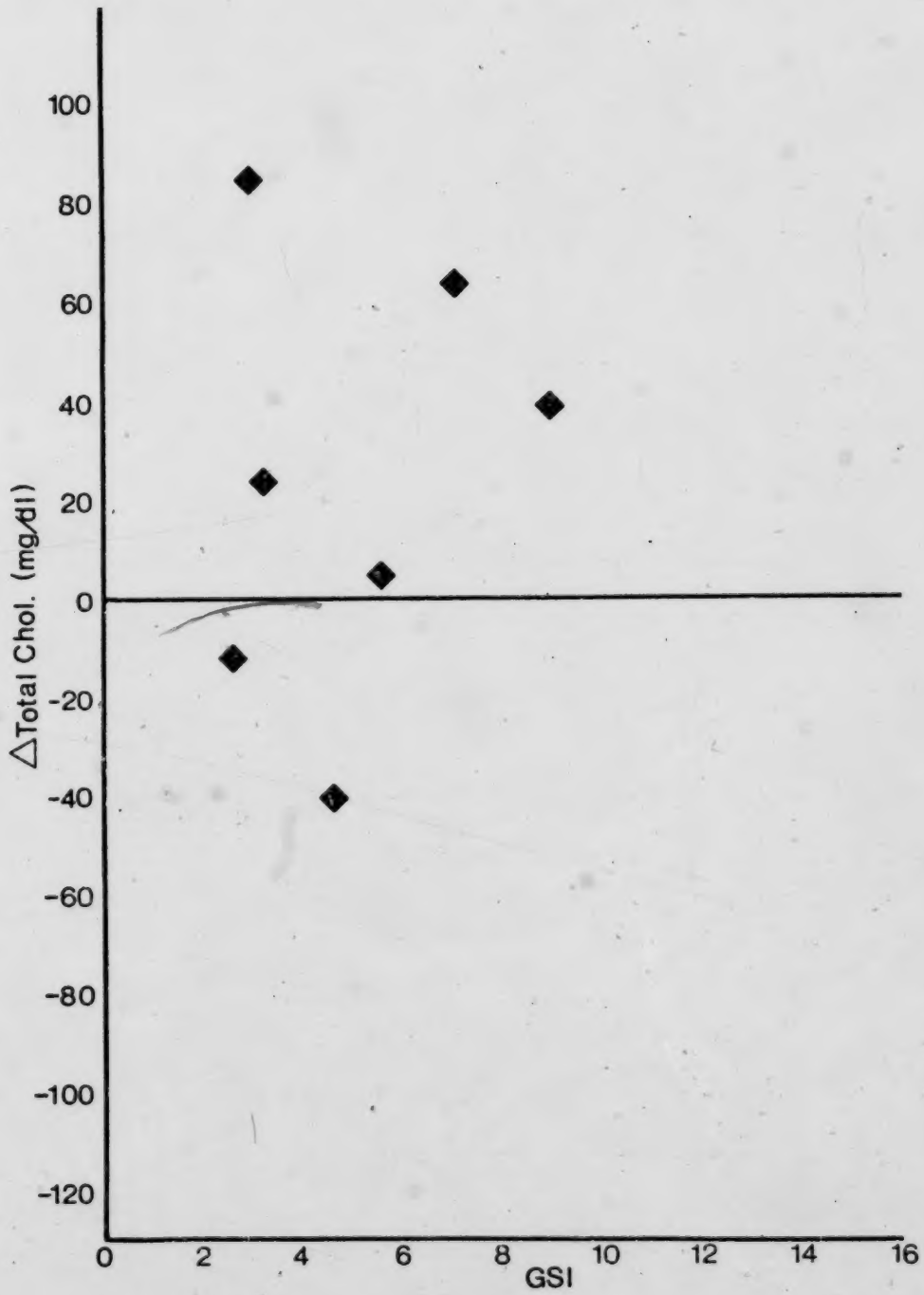

100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
△Total Chol. (mg/dl)
0
2
4
6
8
10
12
14
16
GSI

Figure 10. Regression of changes in plasma TC between samplings on GSI in sexually maturing goldfish (Experiment 1.2) that were uninjected. Symbols as in Figure 8.

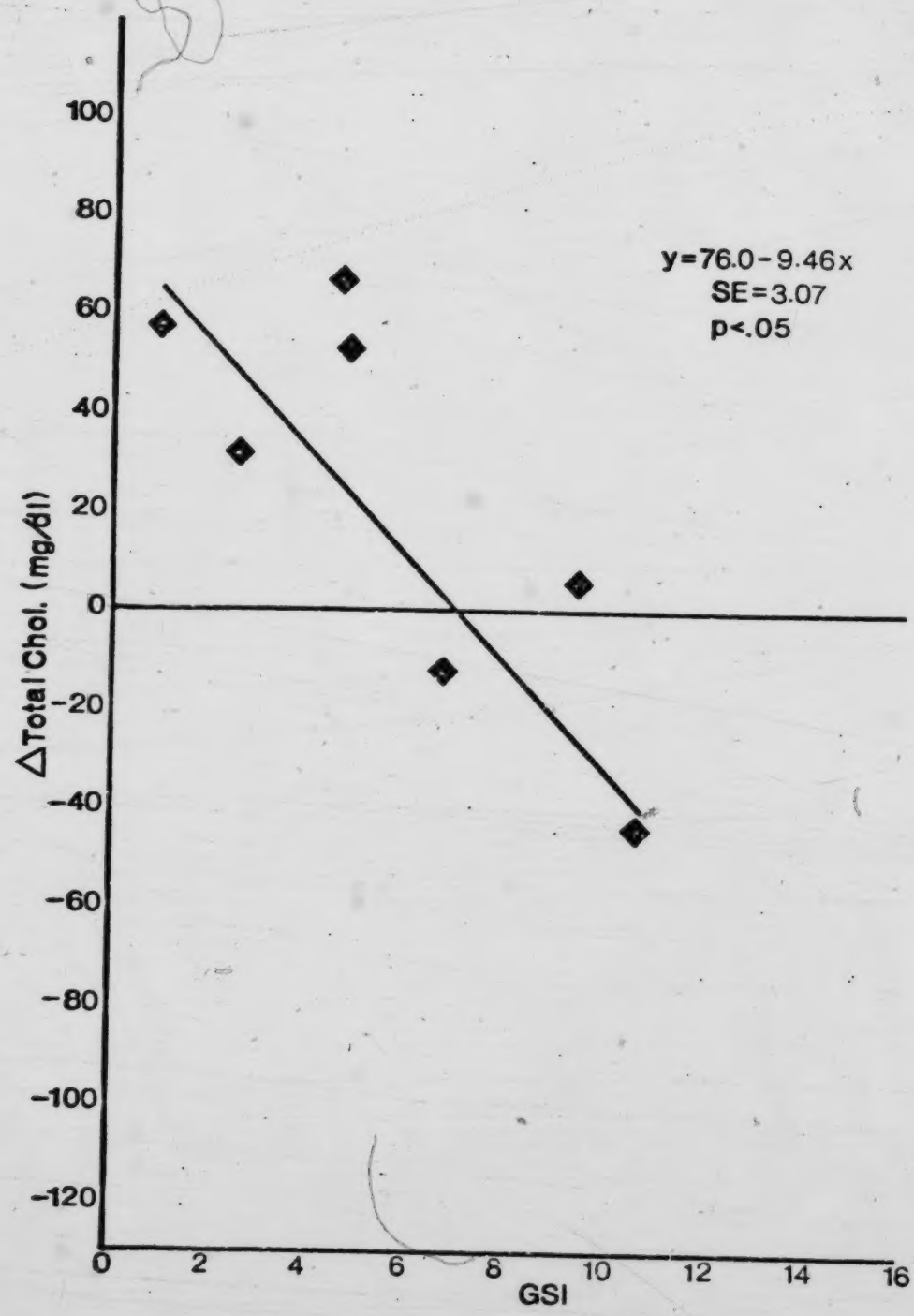

y=76.0-9.46x
SE=3.07
p<.05
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
ΔTotal Chol. (mg/dl)
0
2
4
6
8
10
12
14
16
GSI

TABLE 3. Concentrations of plasma lipid phosphorus, pre-treatment and post-treatment, in Experiments 1.1, 1.3 and 1.4.

| Experiment | Plasma lipid phosphorus (mg/dl) | | | | | |
|---|---|---|---|---|---|---|
| | Treatment Group | | | | | |
| | Uninjected Control | | Injected Control (BSA) | | SG-G100 | |
| | Pre-treatment | Post-treatment | Pre-treatment | Post-treatment | Pre-treatment | Post-treatment |
| 1.1 (Sexually regressed) | 35.37±1.69 | 35.60±1.11 | 35.26±1.40 | 38.01±2.05 | 37.86±1.72 | 40.46±2.02 |
| | N=11 | | N=11 | | N=7 | |
| | n.s. | | $p < .02$ | | n.s. | |
| 1.3 (Sexually mature) | 40.34±1.40 | 39.56±0.94 | 39.79±1.58 | 38.49±0.80 | 37.83±1.19 | 36.44±1.25 |
| | N=12 | | N=11 | | N=7 | |
| | n.s. | | n.s. | | $p < .05$ | |
| 1.4 (Post-ovulatory-regressed) | 34.64±0.84 | 36.19±0.76 | 36.21±1.37 | 39.71±1.45 | 33.16±2.10 | 37.59±1.69 |
| | N=12 | | N=10 | | N=8 | |
| | n.s. | | $p < .001$ | | $p < .02$ | |

NOTE: Mean ± S.E., numbers of fish, and significance of change between samplings, evaluated by paired "$t$" test appear for each group.

n.s. = not significant.

In these experiments there was no significant difference between groups in the mean pre-treatment plasma LP concentrations or between groups in the mean post-treatment plasma LP concentrations (Duncan's test).

Figure 11. Plasma TG in the pre-treatment and post-treatment samples from Experiment 1.5.

Unop. Cont. = Unoperated control goldfish; Uninj. Cast. = Uninjected castrate control goldfish; Inj. Cast. = Castrated control goldfish injected with BSA; SG-G100 = Castrate goldfish injected with SG-G100. Other symbols as in Figure 6.

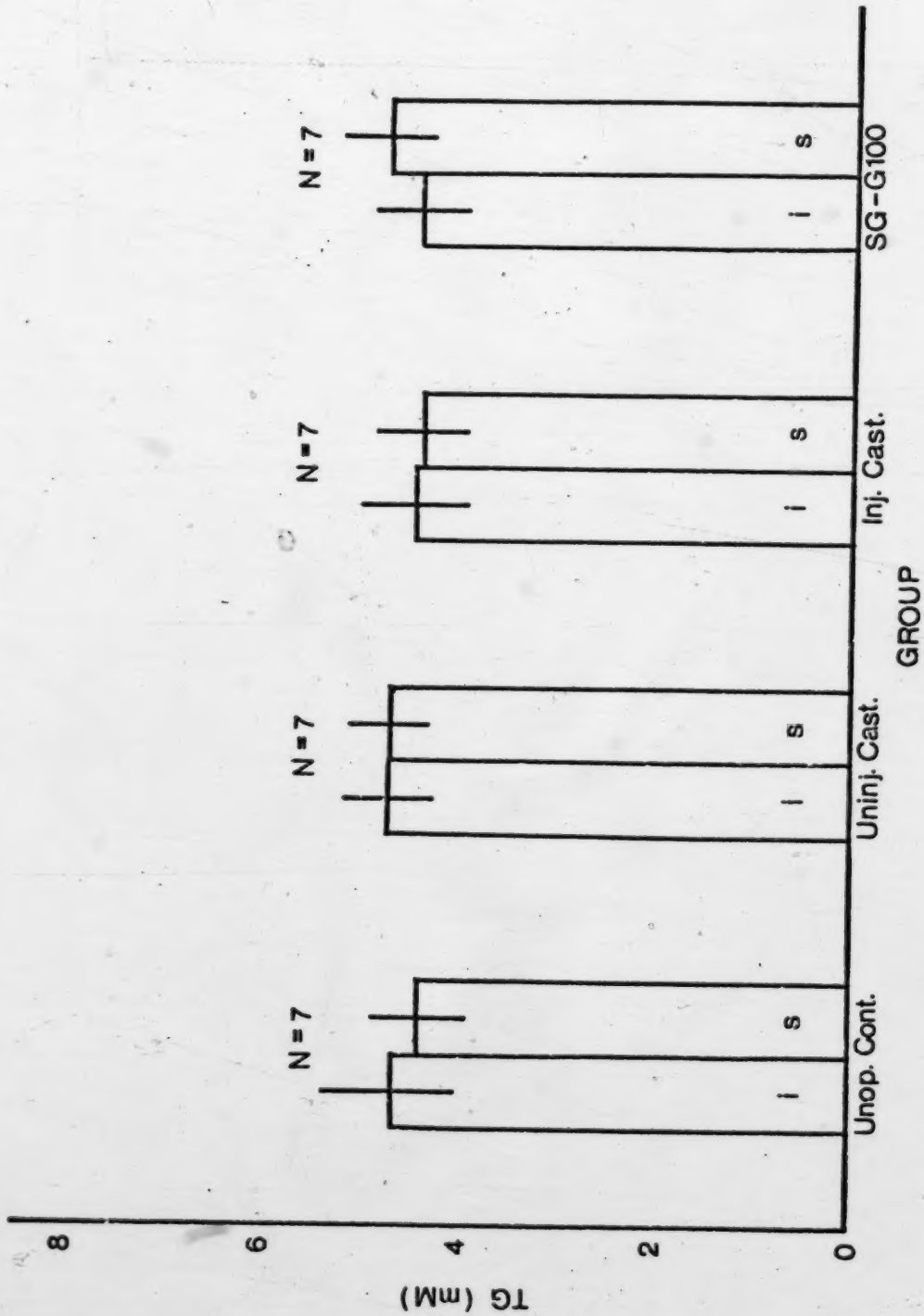
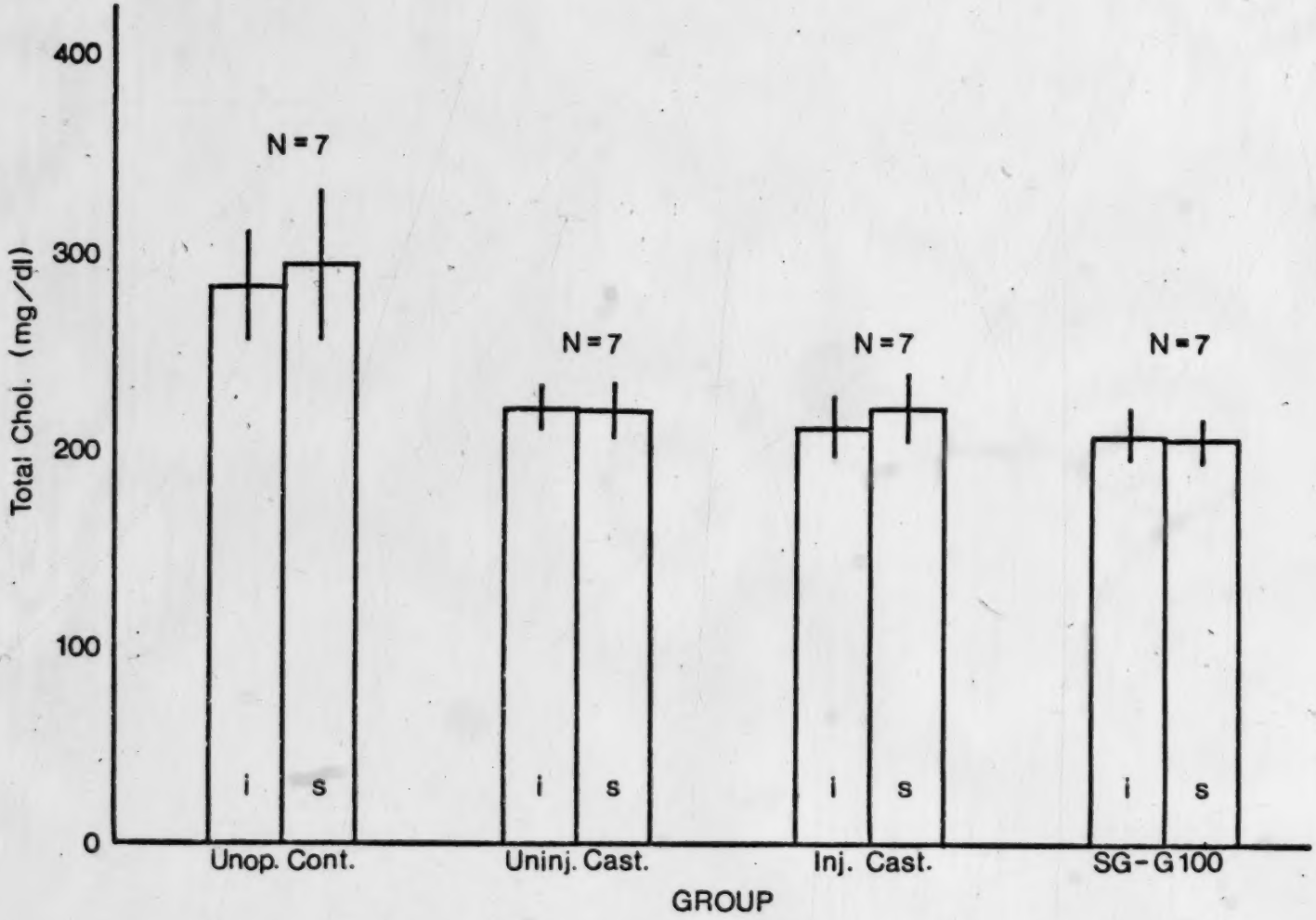

TG (mM)
0
2
4
6
8
N=7
I
S
Unop. Cont.
Uninj. Cast.
Inj. Cast.
SG-G100
GROUP

Figure 12. Plasma TC in the pre-treatment and post-treatment samples from Experiment 1.5. Results of Duncan's test at $p < 0.05$ show that the unoperated controls had higher levels of plasma TC than all other groups in both initial (pre-treatment) and second (post-treatment) samples.

Other symbols as in Figures 2 and 11.


400
300
200
100
0
Total Chol. (mg/dl)
N=7
N=7
N=7
N=7
i
s
i
s
i
s
i
s
Unop. Cont.
Uninj. Cast.
Inj. Cast.
SG-G100
GROUP

## DISCUSSION

In the three experiments conducted with intact fish at 12°C, plasma TG levels in the pre-treatment samples increased with increasing gonad size. If plasma TG is a major source of fatty acids for oxidation by and/or for storage in the developing ovary, higher plasma concentrations of TG in fish with larger ovaries would reflect increased availability of fatty acids for uptake by the ovary. Whatever the metabolic fate of TG, the cause of the increased plasma TG concentrations as ovarian development progresses is probably oestrogen. In support of this, oestrogen injection has been found to cause increased plasma levels of neutral lipid in sockeye salmon, *Oncorhynchus nerka* (Ho and Vanstone 1961) and TG in the bass, *Paralabrax clathratus* (Urist and Schjeide 1961), *S. gairdnerii irideus* (Takashima *et al*, 1972) and goldfish (this thesis, Chapter 2). Plasma oestrogen concentrations have been found to increase with increasing GSI in *Pleuronectes platessa* (Wingfield and Grimm 1977), *Tilapea aurea* (Yaron *et al*. 1977) and *Salmo trutta* (Crim and Idler 1978), but not in *S. gairdneri* (Schreck *et al*. 1973).

It may be that the plasma TG levels in goldfish, as depicted in Fig. 1, are dependent on GSI only up to intermediate values found in maturing fish, and plateau at higher values as the fish reach maturity. If a break is arbitrarily made at GSI = 6 in Fig. 1 and regression equations calculated for fish above and below that boundary, the respective equations are: $y = 2.07 + 0.64x$, S.E. = 0.17, $p < 0.001$, for fish with GSIs less than 6.0, and $y = 4.56 + 0.09x$, S.E. = 0.08, not significant, for fish with GSIs greater than 6.0 (symbols as

above and in figures). Thus, the increase in TG with increasing gonad size may occur only in fish with smaller ovaries. A similar relationship between plasma LP and GSI has been reported in *Platichthys flesus* (Petersen and Emmersen 1977).

Concentrations of plasma TC decreased with increasing GSI in the pre-treatment samples in Experiments 1.1, 1.2 and 1.3. These results have been confirmed in Chapter 2. The relationship described here is similar to, but not as dramatic as, that described by McCartney (1967) in brown trout in which serum cholesterol levels were minimal during the spawning season and maximal one month after its completion. The cause of the decrease is not known and it is probably not mediated through sex steroids (this thesis, Chapter 2). The effects of SG-G100 injection in maturing fish are, however, consistent with a role of gonadotrophin in accelerating cholesterol uptake by the ovary and this could contribute to the decline (see below).

Salmon gonadotrophin was found to increase plasma TG in fish with small ovaries in two experiments (Fig. 3) and to increase plasma TC in similar fish in one experiment (Fig. 8). These increases can probably be attributed to stimulation of ovarian oestrogen secretion by the injected gonadotrophin and oestrogen has been found to increase plasma lipid concentrations in teleosts (for review, see this thesis, Chapter 2). Injection of pituitary extract from vitellogenic sockeye salmon has been shown to increase plasma oestradiol concentration in *S. trutta* (Crim and Idler 1978) and injection of a salmonid gonadotrophin raises plasma oestradiol concentration in rainbow trout (Billard *et al.* 1978). Truscott *et al.* (1978) demonstrated that ovine LH increased plasma testosterone concentrations in gravid catfish,

*Heteropneustes fossilis*. Other evidence for gonadotrophin enchancement of steroid production in fish is indirect. After stimulation by a glycoprotein gonadotrophin isolated from chinook salmon, *Oncorhynchus tshawytscha*, pituitaries, the interstitial cells of the *S. gairdneri* ovary exhibit ultrastructural features normally associated with steroid production (Upadhyay 1977). Sundararaj *et al.* (1972) have associated the proliferation of the follicular layer of the *H. fossilis* ovary, observed after SG-G100 treatment, with increased oestrogen production. Fish gonadotrophins, including SG-G100, have been found to stimulate cyclic AMP production in goldfish ovaries (Fontaine *et al.* 1972), and a chum salmon, *Oncorhynchus keta*, glycoprotein gonadotrophin has been shown to stimulate cyclic AMP production in *S. gairdneri* ovaries (Idler *et al.* 1975). Cyclic AMP is implicated as an intermediary in gonadotrophin-induced steroid production (Marsh 1975; Catt and Dufau 1976).

In Experiments 1.1 and 1.2, there was a transition of the effect of SG-G100 from raising plasma TG in fish with small ovaries to lowering plasma TG in fish with larger ovaries (Fig. 3). A similar effect on plasma TC is apparent in Experiment 1.2 (Fig. 8). The effect of SG-G100 to lower plasma levels of neutral lipids appears to over-ride the oestrogen-mediated action to raise the levels as ovarian growth proceeds. The stimulation by SG-G100 of removal of lipid from the plasma in fish with larger ovaries is likely due to uptake of lipid into the ovary. The transition of the SG-G100 effect on neutral lipids described above could therefore be explained by an increasing capacity of the ovary to take up lipid as ovarian growth proceeds. The deposition of lipid, as judged by histological criteria, has been

demonstrated to accompany accelerated oocyte development in *O. gorbuscha* after SG-G100 injection (Funk *et al.* 1973).

In addition to being dependent on GSI, the effects of SG-G100 on plasma TG were correlated with histological changes in the ovary. The observation of a correlation between a reduction in the number of perinucleolar oocytes and changes in plasma TG following SG-G100 treatment is likely due to recruitment of these oocytes into the more advanced stages of larger, vitellogenic oocytes which accumulate lipid. The relation of changes in plasma TG to increased numbers of secondary and tertiary stage oocytes is consistent with the findings of Lapin (1973) who reported maximal incorporation of TG in the ovary during development of similar stage oocytes in *P. flesus bogdanovi*.

The nature of the gonadotrophin exerting the effects on plasma lipids in this study is not known since the SG-G100 preparation consists of several fractions (Pierce *et al.* 1976). The number and nature of fish gonadotrophins is currently under intensive investigation. As discussed above, glycoprotein gonadotrophins have been implicated in steroidogenesis in teleost ovaries and oestrogen has been shown to raise plasma lipids. Upadhyay (1977) reported the accumulation of lipid bodies by *S. gairdneri* oocytes after treatment *in vivo* with a fish glycoprotein gonadotrophin; however, no accumulation of exogenous yolk (VG) occurred unless a whole pituitary extract was administered. On the other hand, non-glycoprotein gonadotrophins (not adsorbed on concanavalin A-Sepharose) prepared from both the plaice, *Hippoglossoides platessoides* (Campbell and Idler 1976; Bun Ng and Idler 1978a) and *O. keta* (Bun Ng and Idler 1978b) have vitellogenic activity in hypophysectomized winter flounder, *Pseudopleuronectes*

*americanus*. Therefore, while there is support for the involvement of a glycoprotein gonadotrophin in the responses to SG-G100 observed in this study, work with purified preparations of both glycoprotein and non-glycoprotein gonadotrophins is necessary to resolve the issue.

Where SG-G100 had a GSI-dependent effect on plasma lipids (Figs. 3, 8), the corresponding uninjected control fish showed similar changes in lipid levels between samplings (Fig. 5, 10). However, magnitudes of the regression coefficients in Figures 5 and 10 (uninjected fish) were significantly less than those of regression coefficients in Figures 3 and 8 (SG-G100-injected fish), respectively. This suggests that processes occurring in the uninjected fish were magnified by SG-G100 treatment. No GSI-dependent changes in plasma lipids were detected in the BSA-injected controls, possibly reflecting an effect of injection stress.

In the sexually regressed fish at 12°C (Experiment 1.1), there was a significant decrease in plasma TC between samplings in the two control groups but not in the SG-G100-treated fish. This probably does not represent a biological effect of the gonadotrophin since plasma TC levels decreased in 9 of 11 SG-G100-treated fish and there were no differences between groups in post-treatment plasma TC concentration. The reason for the lack of effect of SG-G100 on plasma TC in sexually regressed fish when it affected TG in sexually regressed fish and both TC and TG in sexually maturing fish (Experiment 1.2) is unclear.

The lack of an effect of SG-G100 on plasma lipids in mature females at 12°C (Experiment 1.3) may be due to the deposition of yolk, hence oocyte growth, being largely complete in these fish. There was a significant decrease between samplings in plasma TG in the two

control groups but not in the SG-G100-treated fish in this experiment. However, no biological significance should be attributed to this apparent effect of SG-G100 because there was no difference between groups in mean post-treatment plasma TG concentrations, and 7 of 9 fish in the SG-G100 group showed lower levels of plasma TG post-treatment compared to pre-treatment. SG-G100 did have biological effects in mature fish at 12°C since three fish ovulated and germinal vesicle migration to the periphery of the oocyte was seen in most of the other SG-G100-treated fish.

That the ovaries are required for the response of plasma lipids to SG-G100 treatment at 12°C is supported by the results of Experiment 1.5 in which SG-G100 had no effects on plasma TG or TC in castrate fish. The difference in TC levels between unoperated fish and the 3 groups of castrates may be due to the lack of oestrogen, which has been shown to increase plasma cholesterol levels in teleosts (Ho and Vanstone 1961; this thesis, Chapter 2).

The finding that SG-G100 had no effects on plasma lipids at 21°C (Experiment 1.4), despite a 2.5 fold increase in dose, is in keeping with the opinions of Yamazaki (1965) and Gillet *et al.* (1978) that oocyte development is sensitive to warm temperature in goldfish. Impairment of metabolic responses to sex hormones may be one factor in this sensitivity. That SG-G100 has some biological potency at this temperature was demonstrated by the observation that 3 fish receiving this treatment ovulated.

The slight difference in plasma LP concentrations between sexually regressed and sexually mature fish at 12°C in the pre-treatment samples is in marked contrast to the case in *P. fleusus*

where the concentration of plasma LP in fish at the commencement of the spawning season is approximately 2.5 times that in the post-spawning season (Petersen and Emmersen 1977).

In sexually mature fish at 12°C (Experiment 1.3, Table 3), there were no differences between groups in the post-treatment plasma LP concentrations. In addition, the magnitudes of the decreases in plasma LP between samplings were similar in the SG-G100- and BSA-treated groups. Therefore, it is difficult to attribute biological significance to the statistically significant decrease in plasma LP between samplings in the SG-G100-treated sexually mature fish.

In summary, this study provides data describing the dependence of plasma lipid concentrations in the female goldfish on the reproductive state. Gonadotrophin injection had effects on plasma lipids consistent with induction of both oestrogen-mediated lipid mobilization and with a hormone mediated uptake of lipid by the ovary.

# Chapter 2. EFFECTS OF SEX STEROIDS ON PLASMA TRIGLYCERIDES, CHOLESTEROL AND PHOSPHOLIPIDS IN FEMALE GOLDFISH.

## INTRODUCTION

In earlier studies it has been found that oestrogens induce increased levels of several plasma lipids. Specifically, injection of oestrogen increases plasma concentrations of total lipids and a lipoprotein (de Vlaming *et al.* 1977b) and lipid phosphorus (Bailey 1957) in the goldfish, *Carassius auratus*; phospholipids in the cod, *Gadus morhua* (Plack and Pritchard 1968); total and neutral lipids, free cholesterol and lipid phosphorus in the sockeye salmon, *Oncorhynchus nerka* (Ho and Vanstone 1961); triglycerides, sterols and phospholipids in the bass, *Paralabrax clathratus* (Urist and Schjeide 1961); lipoproteins in the ayu, *Plecoglossus altevelis* (Aida *et al.* 1973a), and a variety of lipids in the trout, *Salmo gairdnerii irideus* (Takashima *et al.* 1972). Oestrogen has also been shown to influence body lipid reserves in the golden shiner, *Notemigonus crysoleucas*, depending on photoperiod regime and treatment dose (de Vlaming *et al.* 1977a). However, in most of the above studies, only one experiment was performed and in no case were temperature effects considered or more than one oestrogen tested.

The work presented in this chapter was undertaken to determine the effects of several sex steroids, including two oestrogens, on three major classes of plasma lipids in female goldfish at different stages of the reproductive cycle and at two temperatures.

# MATERIALS AND METHODS

## I. General Procedures

General procedures for maintenance, injection and blood sampling of fish, lipid analyses and cleaning of glassware were as reported in Chapter 1.

## II. Experiments

### *Standard Experimental Protocol*

Female goldfish were acclimated to conditions of either 12 ± 1°C or 21 ± 1°C and a 12 hours light/12 hours dark (12L/12D) photoperiod for 2 weeks. An initial (pre-treatment) blood sample was then taken. Two weeks were allowed for recovery under the same environmental conditions prior to commencement of hormone or control treatment. In each experiment, one control group was uninjected and another received the injection vehicle. Experimental groups were treated with either oestrone ($E_1$), oestradiol-17β ($E_2$), or testosterone (T) and in 2 experiments a progesterone (P)-treated group was included. The fish were given 5 injections on alternate days (total, 9 days) and on the tenth day, the second (post-treatment) blood sample was taken. Throughout the experiments, the fish were fed twice daily *ad libitum* with Ewos Salmon Grower Extra pellets, size 5P (Ewos Aquaculture International) except for a 24 hour starvation period prior to each blood sampling. After the second sample, the fish were weighed, sacrificed and the ovaries were removed and weighed.

*Specific Experimental Conditions*

*Experiment 2.1*

Female goldfish were held at 28°C - 30°C under simulated natural (Edmonton) photoperiod for 105 days during the summer of 1977, to effect gonadal regression and prevent the onset of recrudescence. The fish were then injected with 100 IU human chorionic gonadotrophin (HCG-Ayerst)/fish on two successive afternoons to cause ovulation of any remaining mature oocytes. However, no ovulated eggs could be stripped from any of the fish on the mornings following HCG injection, indicating that mature oocytes were absent. The fish were kept at 28°C - 30°C for another 8 days after which the temperature was lowered to 12 ± 1°C over a period of 3 days and the fish were then subjected to the standard protocol at 12 ± 1°C and 12L/12D. At this time, the fish weighed 43.8 g (S.D. = 10.2). Upon sacrifice, the mean GSI was 1.1% (S.D. = 0.7). These fish were designated as "sexually regressed".

*Experiment 2.2*

Goldfish (mean weight = 27.1 g, S.D. = 5.6) were subjected to the standard protocol at 12 ± 1°C in January, 1978. Fish that showed signs of illness (N = 12) were discarded from the experiment. Sacrifice of the remainder of the fish (N = 42) in the experiment was delayed until 3 days after the second bleeding to examine fish for symptoms of illness that may have been accelerated by the stress of bleeding. Only fish that appeared healthy at this time (N = 38) were used in the assessment of data. In addition, six other fish were discarded because of sexing error or inability to obtain a sufficiently large blood sample. Upon sacrifice at the end of the experiment, the

mean GSI was 5.8% (S.D. = 3.4); these fish were designated as "sexually maturing".

*Experiment 2.3*

Female goldfish which had previously ovulated were kept at 21 ± 1°C on a simulated natural (Edmonton) photoperiod in June, 1978. On two successive afternoons, the fish were injected with 100 IU HCG (Sigma)/fish and on the subsequent mornings were stripped of ovulated eggs. The temperature was raised to 28°C - 30°C for 2 weeks and the fish were again injected with 100 IU HCG/fish. However, no ovulated eggs could be stripped from the fish on this occasion. The temperature was lowered to 21°C over a period of 2 days. At this time the fish weighed 31.8 g (S.D. = 5.9) and were subjected to the standard protocol at 21 ± 1°C. Upon sacrifice, the mean GSI of the fish was 1.5% (S.D. = 1.1) and they were designated as "post-ovulatory-regressed".

III. Steroid Suspensions

Steroids, purchased from Sigma, were dissolved in a minimal amount of 95% ethanol at 60°C and diluted to volume with the injection vehicle. The vehicle was prepared by dissolving gelatin in double distilled deionized water (DDW). Stock solutions (10:1) of freshwater teleost physiological saline (PS) were then added and the suspensions diluted with DDW to 0.5% gelatin and normal PS. In Experiments 2.1 and 2.2, the concentration of the $E_1$, $E_2$, and T suspensions was 200 µg/ml, and the injection dose was 2.0 µg/2g body weight. In Experiment 2.3, the concentration of $E_1$, $E_2$, and T suspensions was 500 µg/ml, and the injection dose was 5.0 µg/2g body weight. In Experiments 2.2 and 2.3,

the concentration of the P suspension was 2000 µg/ml, and the injection dose was 20 µg/2g body weight. The suspensions were mixed with a vortex mixer prior to injection.

## IV. Statistical Analysis

Analysis of variance and Duncan's multiple range test at either $p < .05$ or $p < .01$ were used to determine the differences between groups in the initial (pre-treatment) or the second (post-treatment) sample (Steel and Torrie 1960). Paired $t$ tests were used to compare plasma lipid concentrations of the pre- and post-treatment samples from individual fish within experimental groups. Unpaired student's $t$ tests were used for comparison of mean plasma lipid concentrations between experiments. Pearson's correlation coefficients and tests of their significance were calculated for pairs of plasma lipids in the pre-treatment samples (Steel and Torrie 1960; Sokal and Rolfe 1969).

# RESULTS

## I. Effects of Sex Steroids

### *Plasma Triglycerides (TG)*

In sexually regressed fish (Experiment 2.1, Fig. 13) there were no significant differences in plasma TG levels between groups at pre-treatment (initial samples). Post-treatment (second samples), all three groups of steroid-treated fish had higher levels ($p < 0.05$) of plasma TG than the vehicle-injected controls, and the $E_1$-treated group had higher ($p < 0.01$) levels of TG than both control groups. The uninjected control group showed a significant decrease in plasma TG levels between samplings, and the $E_1$-treated group had a significant increase in plasma TG between samplings.

In sexually maturing fish (Experiment 2.2, Fig. 14), there were no significant differences in plasma TG levels between groups at pre-treatment. Post-treatment, the P-treated group had significantly higher ($p < 0.05$) plasma TG concentrations than either the vehicle- or T-treated fish. The T-treated fish showed a significant decrease in plasma TG between samplings.

In the post-ovulatory-regressed fish (Experiment 2.3, Fig. 15), there were no significant differences between groups in either the pre- or post-treatment samples. Plasma TG levels increased in both the T- and P-injected fish between samplings.

### *Plasma Total Cholesterol (TC)*

In the sexually regressed fish (Fig. 16), the pre-treatment level of TC in the vehicle-injected fish was significantly lower than in the

Figure 13. Plasma triglycerides (TG) of sexually regressed goldfish (Experiment 2.1). Bars represent mean ± SE for the pre-treatment, or initial (i) sample, and post-treatment or second (s) sample from each group. Numbers of fish (N) and results of paired *t* test, if significant, appear above each pair of bars. Results of Duncan's new multiple range test on post-treatment (second) samples are inset, if significant; groups underlined by the same line are not different at the indicated level of significance.

Un = Uninjected control goldfish; V = Goldfish injected with vehicle; T, E1, E2 = goldfish injected with testosterone, oestrone and oestradiol-17β, respectively.

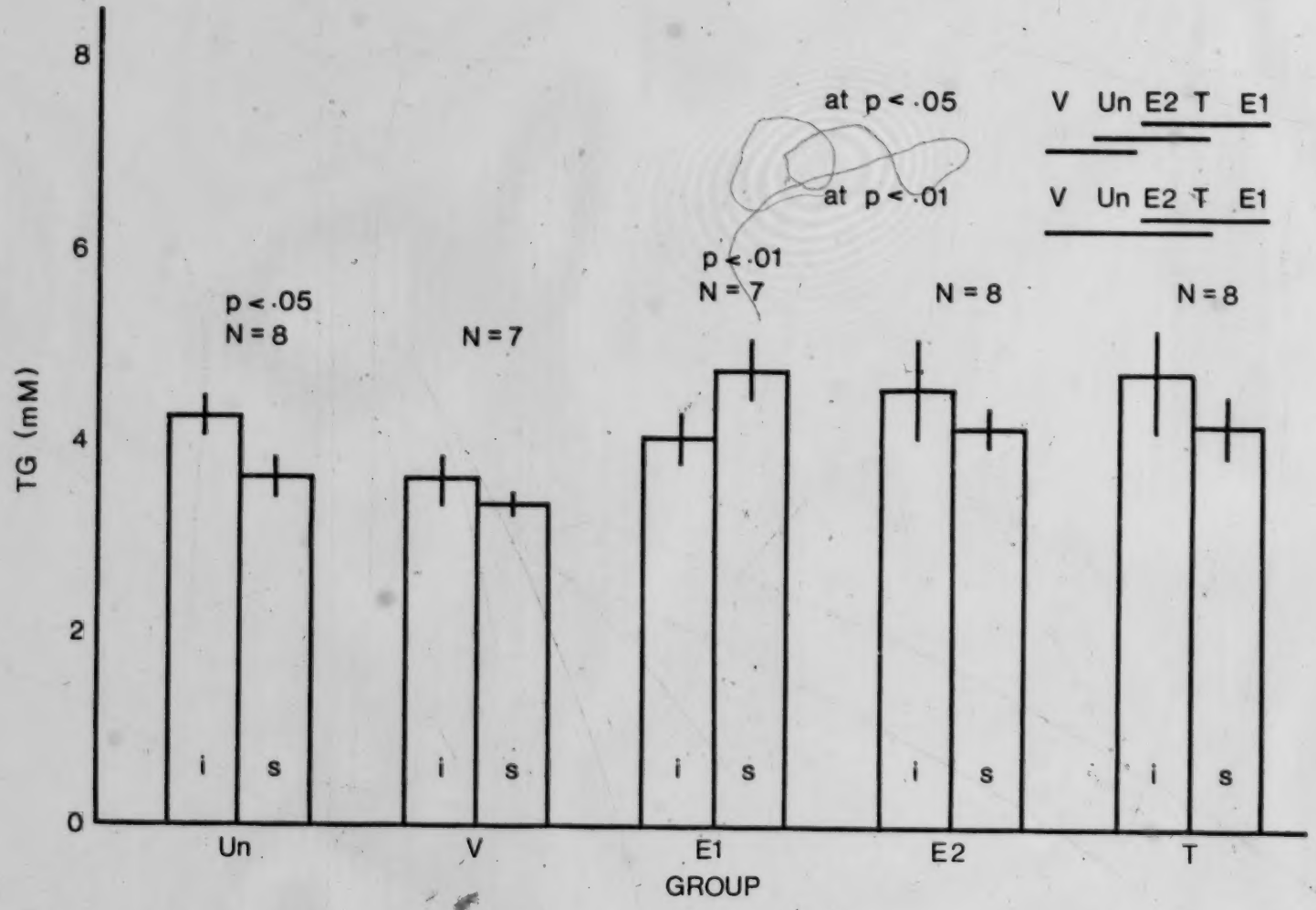

8
6
4
2
0
TG (mM)
at p < .05
V Un E2 T E1
at p < .01
V Un E2 T E1
p < .05
N = 8
N = 7
p < .01
N = 7
N = 8
N = 8
i
s
Un
V
E1
E2
T
GROUP

Figure 14. Plasma TG of sexually maturing goldfish (Experiment 2.2).

P = goldfish injected with progesterone.

Other symbols and details as in Figure 13.

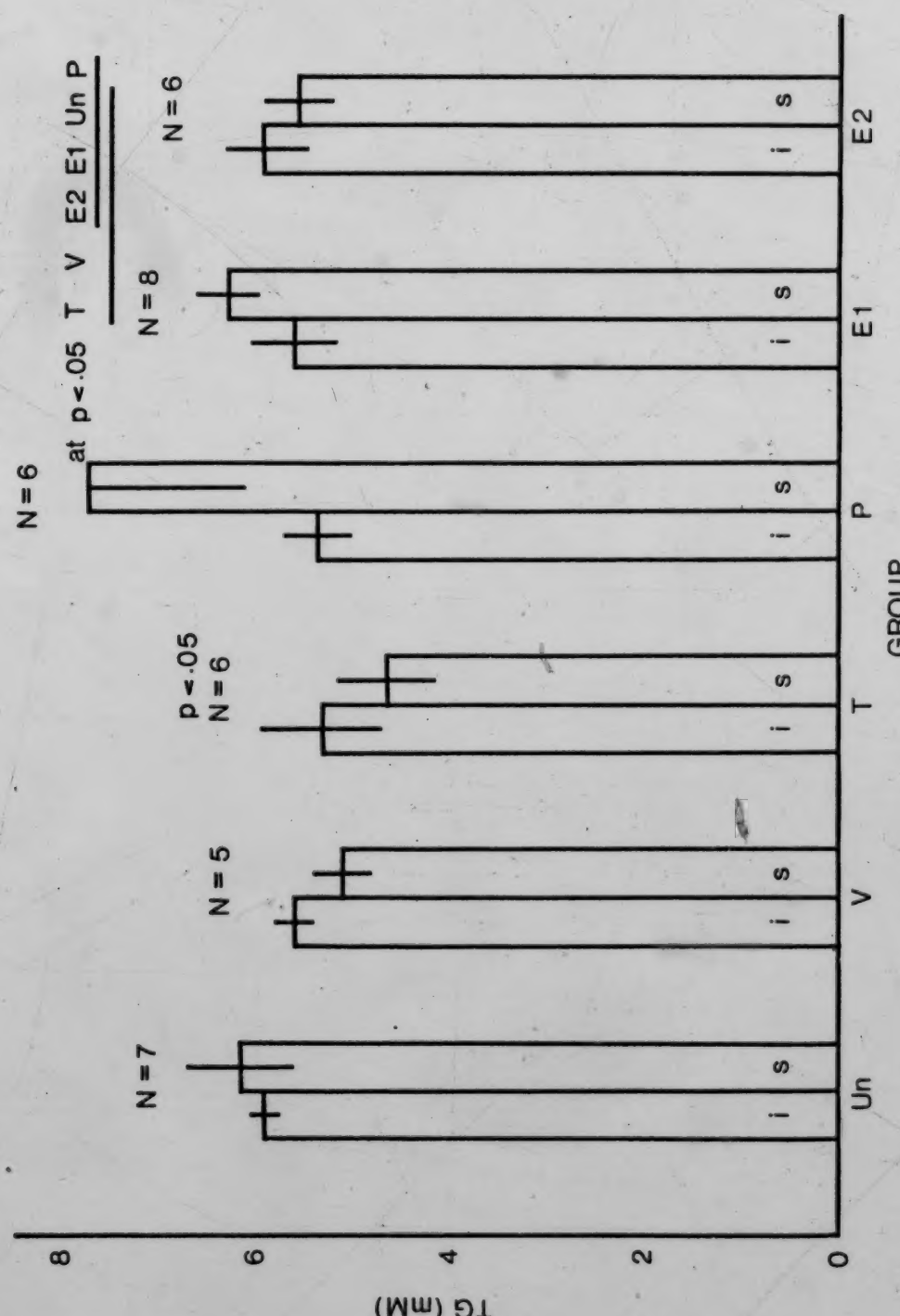

TG (mM)
8
6
4
2
0
Un
V
T
P
E1
E2
GROUP
i
s
N=7
N=5
p<.05
N=6
N=6
at p<.05 T V E2 E1 Un P
N=8
N=6

Figure 15. Plasma TG of post-ovulatory regressed goldfish (Experiment 2.3).

Symbols and other details as in Figures 13 and 14.

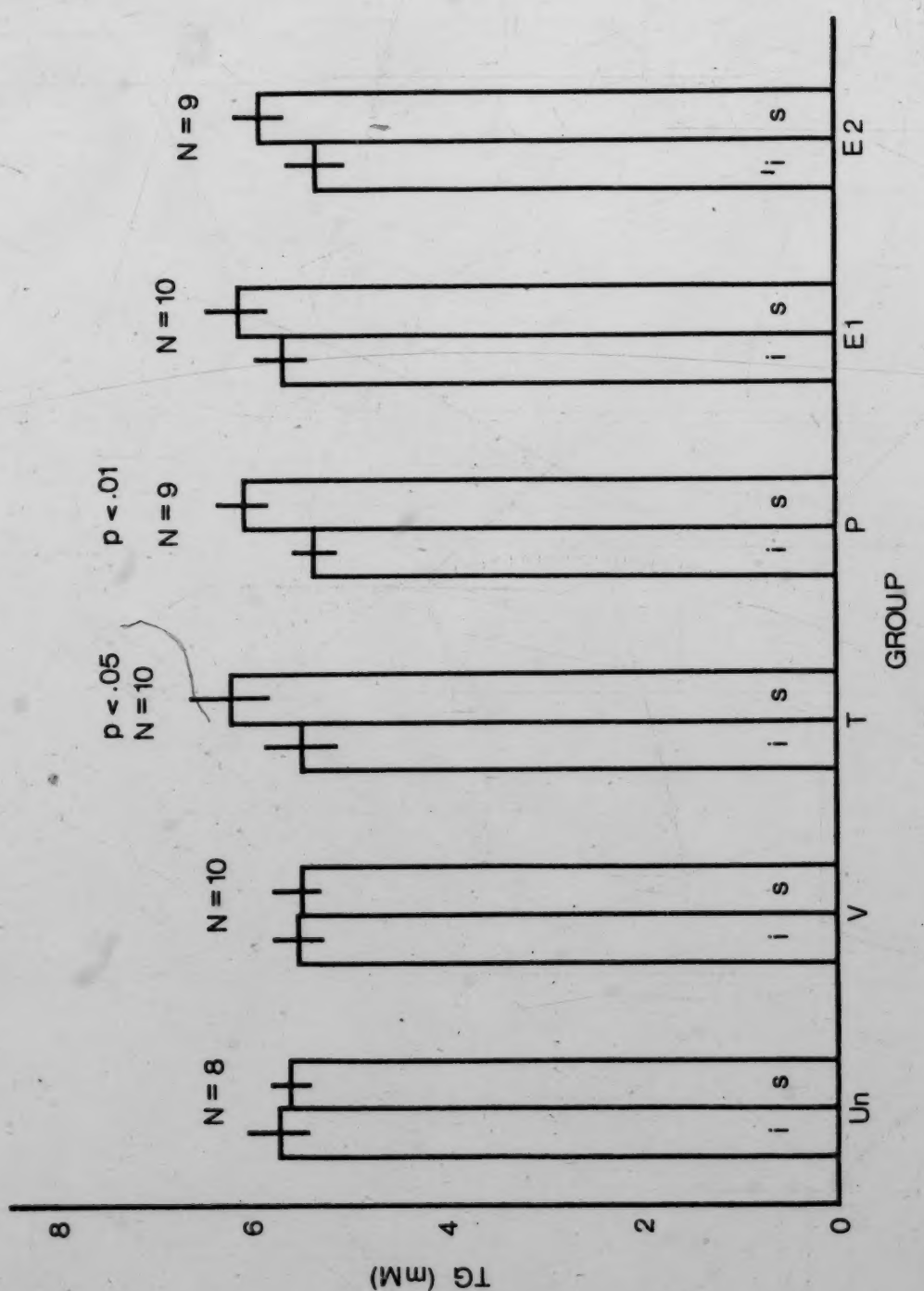
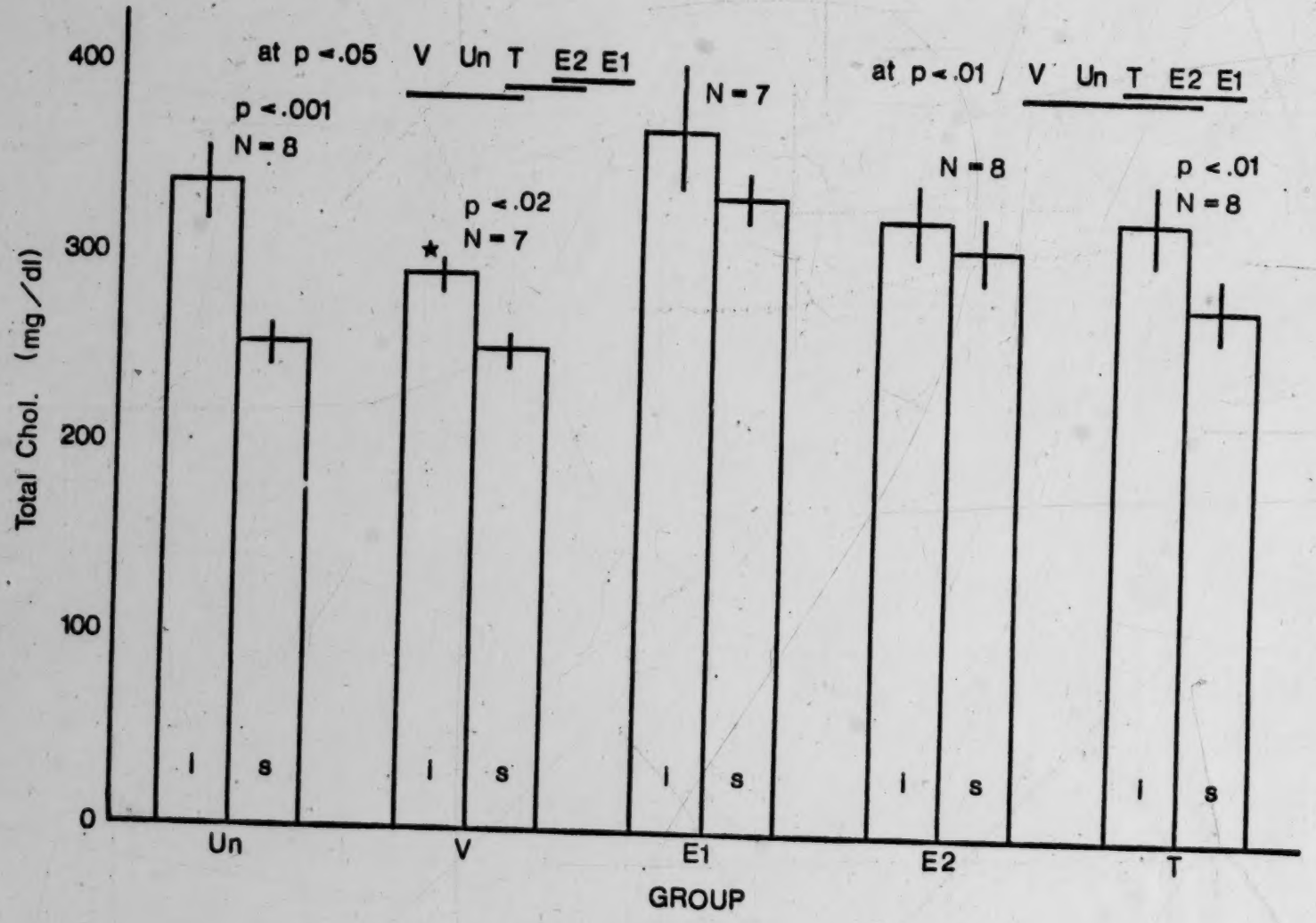

TG (mM)
8
6
4
2
0
Un
V
T
P
E1
E2
GROUP
i
s
N=8
N=10
N=10
N=9
N=10
N=9
p < .05
p < .01

Figure 16. Plasma total cholesterol (TC) of sexually regressed goldfish (Experiment 2.1). *Pre-treatment levels in the V group were less than in the E1 group (Duncan's test, $P < 0.05$).

Total Chol. = Plasma TC. Other symbols and details as in Figure 13.


400
300
200
100
0
Total Chol. (mg/dl)
at p <.05 V Un T E2 E1
at p <.01 V Un T E2 E1
p <.001
N = 8
p <.02
N = 7
N = 7
N = 8
p <.01
N = 8
I
S
Un
V
E1
E2
T
GROUP

$E_1$-injected fish ($p < 0.05$). In the post-treatment samples, the $E_1$-treated fish had higher concentrations of plasma TC than the two control groups ($p < 0.01$) and the T-treated fish ($p < 0.05$), and the $E_2$-treated fish had higher plasma TC levels than the two control groups ($p < 0.05$). Between samplings, there were significant decreases in plasma TC in the uninjected and the vehicle- and T-injected fish.

In the sexually maturing fish (Fig. 17), there were no significant differences between groups in plasma TC levels at pre-treatment. Post-treatment, the $E_1$-treated fish had higher plasma TC levels than the vehicle-injected control group ($p < 0.05$), and the T-treated and uninjected control groups ($p < 0.01$). Levels of TC in the uninjected control group were also less than in the P-treated fish ($p < 0.05$) and the $E_2$-treated fish ($p < 0.01$). Plasma TC levels increased significantly in the P-, $E_1$- and $E_2$-treated fish between samplings.

In the post-ovulatory-regressed fish (Fig. 18), there were no significant differences in plasma TC between groups at either pre- or post-treatment. Plasma TC increased between samplings in both the vehicle- and $E_1$-treated groups.

*Plasma Lipid Phosphorus (LP)*

In the sexually regressed fish (Fig. 19), the pre-treatment concentration of plasma LP in the $E_1$-treated group was significantly higher than in the vehicle- ($p < 0.01$) and the $E_2$- ($p < 0.05$) treated fish. Post-treatment, plasma LP was significantly higher in both $E_1$- and $E_2$-treated groups than in the other 3 groups ($p < 0.01$). The $E_1$-treated fish also had higher plasma LP levels than the $E_2$-treated fish, and plasma LP was higher in the T- than in the vehicle-injected fish ($p < 0.05$). Plasma LP increased significantly between samplings

Figure 17. Plasma TC of sexually maturing goldfish (Experiment 2.2).

Symbols and other details as in Figures 13 and 14.

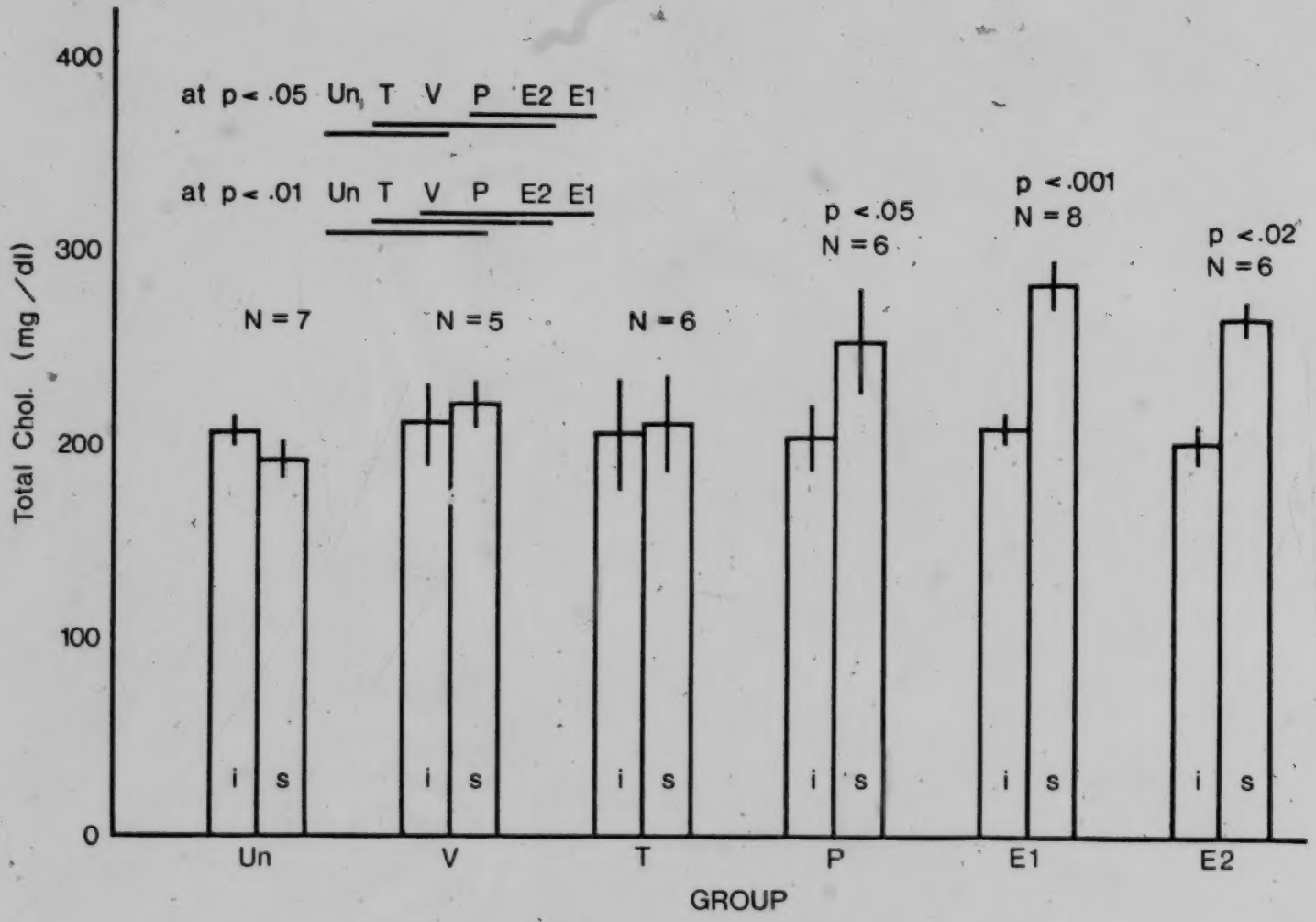

400
300
200
100
0
Total Chol. (mg/dl)
at p< .05 Un T V P E2 E1
at p< .01 Un T V P E2 E1
N = 7
N = 5
N = 6
p <.05
N = 6
p <.001
N = 8
p <.02
N = 6
i
s
Un
V
T
P
E1
E2
GROUP

Figure 18. Plasma TC of post-ovulatory regressed goldfish (Experiment 2.3).

Symbols and other details as in Figures 13 and 14.

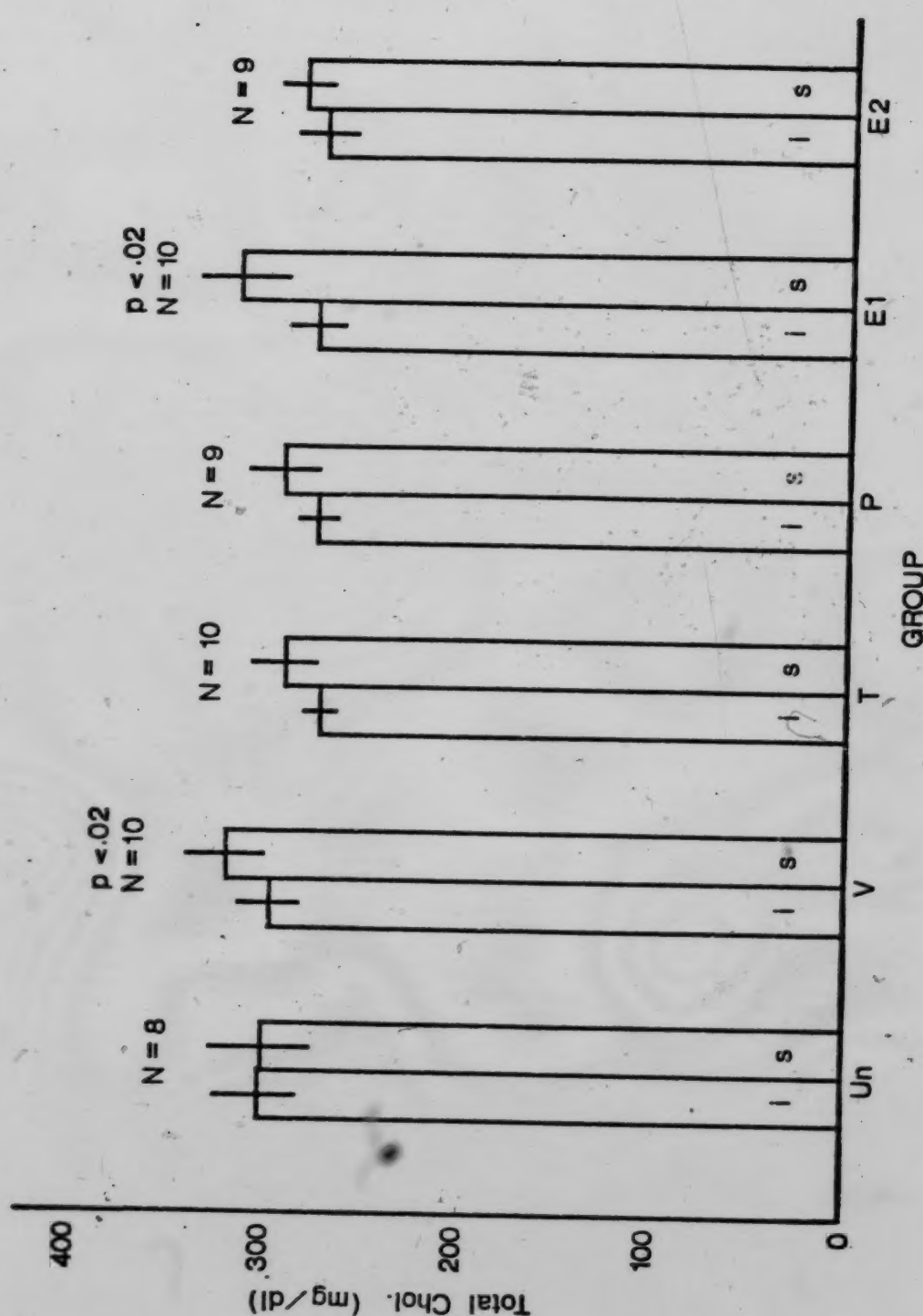
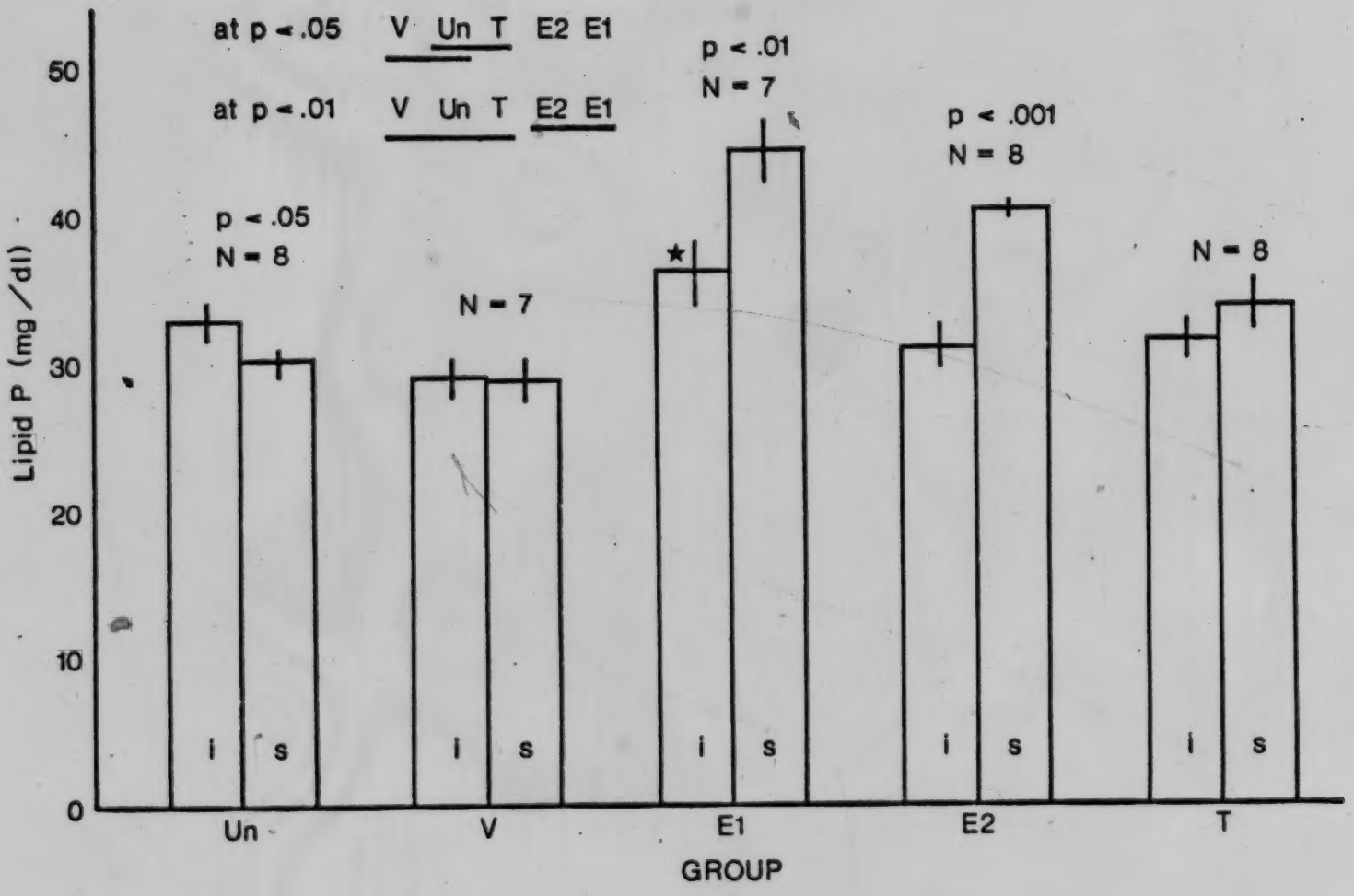

Total Chol. (mg/dl)
400
300
200
100
0
N = 8
p < .02
N = 10
N = 10
N = 9
p < .02
N = 10
N = 9
Un
V
T
P
E1
E2
GROUP
I
S

Figure 19. Plasma lipid phosphorus (LP) of sexually regressed goldfish (Experiment 2.1). *Pre-treatment levels of LP in the E1 group were significantly higher than in the V group (Duncan's test, $p < 0.01$) and E2 group (Duncan's test, $p < 0.05$).
Lipid P = Plasma LP. Other symbols and details as in Figure 13.


at p < .05 V Un T E2 E1
at p < .01 V Un T E2 E1
p < .05
N = 8
N = 7
p < .01
N = 7
p < .001
N = 8
N = 8
Lipid P (mg/dl)
50
40
30
20
10
0
i
s
Un
V
E1
E2
T
GROUP

in both oestrogen-treated groups and decreased in uninjected fish.

In the sexually maturing fish (Fig. 20), there were no significant differences in plasma LP levels between groups at pre-treatment. Post-treatment, the concentrations of plasma LP in both groups of oestrogen-treated fish were greater than in all the other groups ($p < 0.01$). Both oestrogen-treated groups showed increased levels of LP at post-treatment compared to pre-treatment.

The results with the post-ovulatory-regressed fish at 21 ± 1°C for plasma LP (Fig. 21) were similar to those in the experiment with the maturing fish at 12 ± 1°C (Fig. 20). The post-treatment levels of plasma LP in the oestrogen-treated fish were higher than in all the other groups ($p < 0.01$) and significantly increased over pre-treatment levels ($p < 0.001$).

II. Pre-treatment Plasma Lipid Concentrations

Comparison of the pre-treatment concentrations of plasma lipids in the two experiments conducted at 12 ± 1°C showed that the levels of plasma TG from the sexually regressed fish (Experiment 2.1) were significantly lower than in the sexually maturing fish (Experiment 2.2) (4.27 mM ± 0.17 [SEM] vs. 5.61 ± 0.16 mM, $p < 0.001$) and that the pre-treatment levels of TC in the sexually regressed fish were higher than in the maturing fish (329 ± 10 mg/dl vs. 208 ± 6 mg/dl, $p < 0.001$). Pre-treatment levels of TG in the sexually maturing fish (Experiment 2.2) were higher than the post-treatment levels of TG in the $E_1$-treated group from Experiment 1 ($p < 0.02$) and pre-treatment levels of TC in the sexually maturing fish were lower than post-treatment levels in control fish from Experiment 2.1 ($p < 0.001$). There were no differences

Figure 20. Plasma LP of sexually maturing goldfish (Experiment 2.2).

Symbols and other details as in Figures 13, 14 and 19.

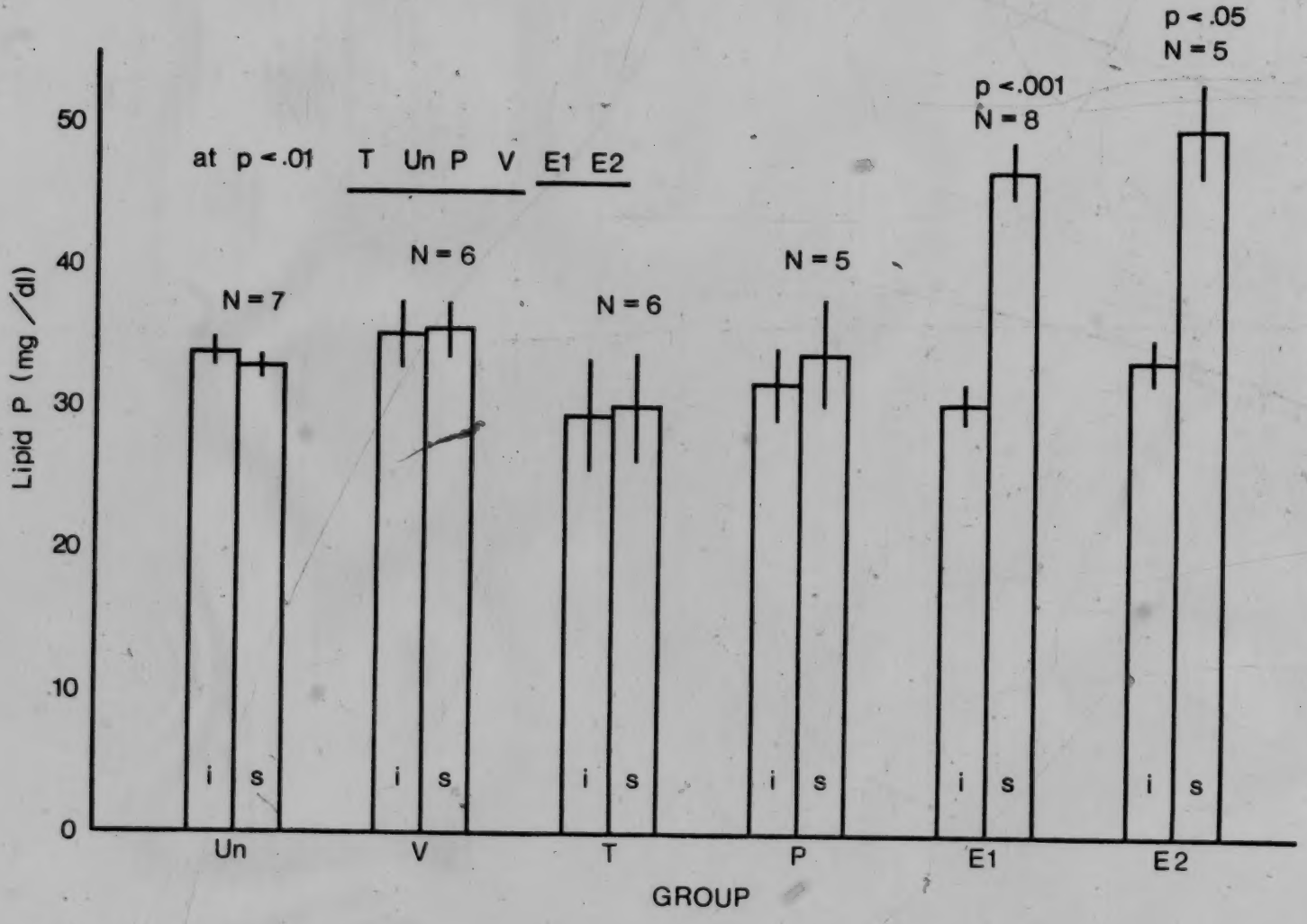
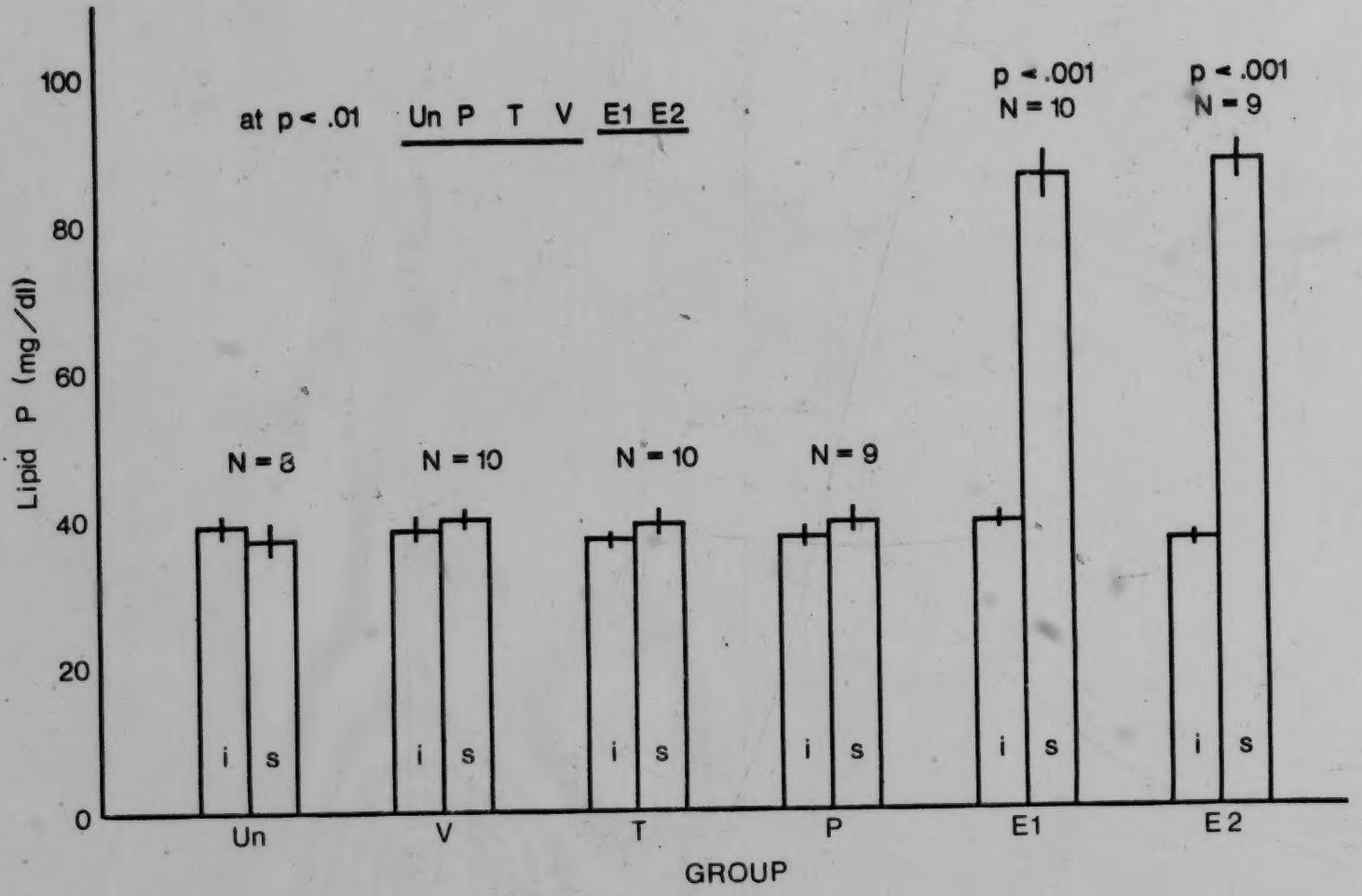

p < .05
N = 5
p < .001
N = 8
at p < .01 T Un P V E1 E2
N = 7
N = 6
N = 6
N = 5
Lipid P (mg / dl)
50
40
30
20
10
0
i s
Un
V
T
P
E1
E2
GROUP

Figure 21. Plasma LP of post-ovulatory regressed goldfish (Experiment 2.3).

Symbols and other details as in Figures 13, 14 and 19.


at p < .01 Un P T V E1 E2
p < .001
N = 10
p < .001
N = 9
N = 8
N = 10
N = 10
N = 9
Lipid P (mg/dl)
100
80
60
40
20
0
i
s
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E1
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between the pre-treatment concentrations of plasma LP in the two experiments.

Comparison of the pre-treatment plasma lipid concentrations in Experiments 2.1 and 2.3, in which the fish had similar GSIs, revealed that the pre-treatment plasma TG levels were significantly higher in the post-ovulatory-regressed fish at 21 ± 1°C (5.50 ± 0.11 mM) than in the sexually regressed fish at 12 ± 1°C (4.27 ± 0.17 mM) ($p < 0.001$). Pre-treatment plasma LP levels were also significantly higher in the post-ovulatory-regressed fish at 21 ± 1°C than in the regressed fish at 12°C (37.8 ± 0.5 mg/dl vs. 32.1 ± 0.8 mg/dl, $p < 0.001$), but pre-treatment plasma TC concentrations were lower in the post-ovulatory-regressed fish at 21 ± 1°C than in the regressed fish at 12 ± 1°C (282 ± 6 mg/dl vs. 329 ± 10 mg/dl, $p < 0.001$).

Pairs of plasma lipids from the pre-treatment samples were assessed for significant correlations (Table 4). In all three experiments, there was a highly significant correlation between pre-treatment levels of LP and TC. Pre-treatment levels of plasma TG significantly correlated with levels of LP in all three experiments but with TC only in maturing fish.

TABLE 4. Correlation coefficients ($r$) for pre-treatment plasma concentrations of pairs of lipids in Experiments 2.1, 2.2 and 2.3.

| Experiment | TC vs. LP | TG vs. LP | TG vs. TC |
|---|---|---|---|
| 2.1 (Sexually regressed) | $r$ = .780<br>N = 38<br>$p$ < 0.001 | $r$ = .366<br>N = 38<br>$p$ < 0.05 | $r$ = .102<br>N = 38<br>n.s. |
| 2.2 (Sexually maturing) | $r$ = .645<br>N = 36<br>$p$ < 0.001 | $r$ = .627<br>N = 36<br>$p$ < 0.001 | $r$ = .405<br>N = 38<br>$p$ < 0.02 |
| 2.3 (Post-ovulatory-regressed) | $r$ = .444<br>N = 56<br>$p$ < 0.001 | $r$ = .580<br>N = 56<br>$p$ < 0.001 | $r$ = -.109<br>N = 56<br>n.s. |

## DISCUSSION

The results of this study provide support for the hypothesis that oestrogen is involved in lipid mobilization in teleosts. Injection of $E_1$, but not $E_2$, caused a significant increase in plasma TG levels in sexually regressed fish at 12°C (Experiment 2.1). The increase in plasma TG after $E_1$ injection is possibly due to increased liver release of this lipid. The liver of *N. crysoleucas* has been shown to release TG *in vitro* (Pardo and de Vlaming 1976). An effect of $E_1$ on release of TG by the liver of a teleost has not been reported, to my knowledge. De Vlaming *et al.* (1977a) could not demonstrate an effect of $E_2$ treatment of *N. crysoleucas* liver slices to increase medium TG concentrations, even when the fish had been primed with $E_2$. This is consistent with the lack of effect of $E_2$ on plasma TG in the goldfish in the present study.

The mean pre-treatment concentration of plasma TG in sexually maturing fish at 12°C (Experiment 2.2) was higher than that in the sexually regressed fish at 12°C (Experiment 2.1), and was also higher than the post-treatment concentration of plasma TG in the $E_1$-treated fish from Experiment 2.1. The higher pre-treatment levels in the maturing fish are likely due to higher endogenous oestrogen levels as compared to the regressed fish (see this thesis, Chapter 1 for review).

That $E_1$, but not $E_2$, raised plasma TG in sexually regressed fish at 12°C is of particular interest. In a pilot experiment leading to the present work, female goldfish were implanted intraperitoneally with

Silastic$^{R}$ capsules containing oestrogen[1] and it was also found that fish implanted with $E_1$, but not those implanted with $E_2$, had higher plasma TG levels than those with control implants. In *Xenopus laevis*, it was found that the relative potencies of oestrogens in the raising of plasma levels of a number of lipid and protein parameters were $E_2 > E_1 >$ oestriol ($E_3$) (Redshaw *et al.* 1969). In *Tilapia aurea*, $E_3$ was found to be more potent than either $E_1$ or $E_2$ in the raising of plasma calcium and total protein concentrations (Terkatin-Shimony and Yaron 1978). Although these studies contradict the present findings, they are not directly comparable to the present study in terms of species or parameters.

Treatment of sexually maturing fish at 12°C with P caused a mean post-treatment concentration of plasma TG that was higher than in the vehicle-injected controls, although the change between samplings in the P-treated group was not significant (paired *t* test). In rats, P has been found to activate lipoprotein lipase (LPL), an important enzyme in the process of removal of TG from the plasma (Kim and Kalkhoff 1975; Valette *et al.* 1978) and to prevent the oestrogen-induced increase in plasma TG (Valette *et al.* 1978). In hyperlipoproteinaemic women, administration of a progestational analogue reduced plasma TG and increased plasma LPL activity (Glueck *et al.* 1969). If a mammalian-like system were operative in goldfish, P treatment of sexually maturing fish

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1 Capsules were made with Silastic Medical-Grade Tubing (Dow Corning) which is permeable to steroids.

might be expected to lower plasma TG since these fish had elevated levels of plasma TG in the pre-treatment sample. The results of Experiment 2.2 are not consistent with such a mechanism.

Whether or not the high level of TG induced by P is due to subsequent conversion of P to other steroids is not known. Conversion of P to T (Colombo and Belvedere 1976) and T to $E_2$ (Lambert *et al.* 1971) have been reported in teleost ovarian preparations. However, in the catfish, *Heteropneustes fossilis*, P has been shown to have a slightly androgenic effect (Sundararaj and Goswami 1968). In this experiment, the effect of P was opposite to that of T, making its action by conversion to T unlikely.

Treatment with T lowered plasma TG in sexually maturing fish at 12°C, although post-treatment levels were not different from those in the vehicle-injected controls. This result is consistent with the report of Schjeide and Lai (1970) that T reduces the release of lipids from the liver of chickens in the presence of oestrogens.

In sexually regressed fish at 12°C (Experiment 2.1), injection of either $E_1$ or $E_2$ prevented the significant decline in plasma TC that occurred between samplings in the other three groups. The reason for this decline is unknown, although it should be noted that the post-treatment levels in the control fish in Experiment 2.1 were still higher than the pre-treatment levels in the sexually maturing fish at 12°C (Experiment 2.2). In Experiment 2.2, treatment with $E_1$, $E_2$ or P caused a significant increase in plasma TC, and $E_1$ caused post-treatment levels that were higher than those in the vehicle-injected controls. $E_1$ was therefore marginally more effective than $E_2$. These results support the findings of other workers that oestrogen is

involved with the mobilizing of cholesterol in teleosts (Ho and Vanstone 1961; Urist and Schjeide 1961). The effect of P has not previously been reported, to my knowledge.

The action of steroids to mobilize cholesterol would enhance availability of cholesterol to the ovary for uptake. The effects of steroids on plasma TC do not offer an explanation for the decline of plasma TC that has been observed to accompany ovarian growth in brown trout (McCartney 1967), *O. nerka* (Idler and Tsuyuki 1958), catfish, *Heteropneustes fossilis* (Singh and Singh 1979) or goldfish (this thesis, Chapter 1 and above). However, gonadotrophin has been implicated in the uptake of cholesterol and other lipid from the plasma by the ovary (this thesis, Chapter 1).

In the sexually regressed and sexually maturing fish at 12°C and in post-ovulatory fish at 21°C, injection of either $E_1$ or $E_2$ elevated plasma LP concentrations, producing post-treatment levels that were higher than in the controls. Both T and P were without effect. These results support the hypothesis that oestrogen is involved in phospholipid mobilization in teleosts (Bailey 1957; Ho and Vanstone 1961; Urist and Schjeide 1961). Post-treatment plasma LP concentrations in the oestrogen-treated post-ovulatory-regressed fish at 21°C were approximately double those in the corresponding fish in two experiments at 12°C. Whether or not this represents a potentiation of this response to oestrogen at higher temperatures is not known since the hormone dose was higher in the 21°C experiment by a factor of 2.5.

Injection of oestrogens into post-ovulatory-regressed fish at 21°C (Experiment 2.3) had no effect on plasma TC or TG, but both oestrogens raised plasma LP concentrations. Therefore some, but not

all, metabolic effects of oestrogen were abolished at higher temperature, despite a 2.5-fold increase in oestrogen dose. As suggested in the previous chapter, it is possible that higher temperatures are inhibitory to the metabolic processes stimulated by sex hormones. Yamazaki (1965) and Gillet *et al.* (1978) have suggested that oocyte development is sensitive to high temperature in this species.

In summary, the data presented in this chapter support the hypothesis that oestrogen is involved in lipid mobilization in teleosts. $E_1$ was shown to be equipotent to, or more potent than $E_2$ in the raising of plasma concentrations of lipids. There was no support for mammalian-like stimulation by P of removal of TG from the plasma. The effects of oestrogen on plasma TC and TG were abolished at 21°C but the effect on plasma LP was not.

# Chapter 3. EFFECTS OF SEX STEROIDS AND STARVATION ON PLASMA FREE FATTY ACIDS IN THE GOLDFISH.

## INTRODUCTION

The depletion of body fat reserves has been associated with the provision of energy for gonad growth in a number of teleost species (for review, see General Introduction). Triglyceride (TG) is the stored lipid most extensively depleted during gametogenesis in the cod, *Gadus morhua callarias* (Shatunovskiy 1971) and the scorpionfish, *Scorpaena porcus* (Shchepkin 1971). When stored TG is mobilized for oxidation by other tissues in mammalian systems, free fatty acids (FFA) are released into the plasma (Newsholme and Start 1973). The importance of plasma FFA to gonad recrudescence in teleosts is unknown but, if plasma FFA are utilized in gonad growth, it could be expected that sex steroids would influence their levels.

Oestrogen has been shown to raise plasma FFA concentrations in the amphibian, *Xenopus laevis* (Follett and Redshaw 1968), the reptile, *Anolis carolinensis* (Gist and Rainey 1975), domestic fowl (Heald and Rookledge 1964), 12-hour fasted rats (Kim and Kalkhoff 1975) and the teleost, *Platichthys flesus* (Petersen and Korsgaard 1978). Preliminary studies on the goldfish indicated that injection of either oestradiol-17β ($E_2$) or testosterone (T) caused elevated plasma FFA levels but that such effects were sensitive to the length of fast prior to blood sampling.

The mechanism by which sex steroids could act to raise plasma FFA levels is not known. $E_2$ mobilizes FFA in the fat body of the reptile,

*Uta stansburiana* but does not have direct lipolytic activity when applied directly to the fat body (Hahn 1967). It was considered possible that sex steroids could cause elevated plasma FFA levels by acting at the hypothalamus to influence the secretion of pituitary hormones. Uptake of sex steroids by the teleost hypothalamus has been reported ( Kim *et al.* 1978; Morrell and Pfaff 1978) and several mammalian pituitary hormones or hormones secreted from their target glands have been shown to enhance lipolysis (Goodman 1970; Kastin *et al.* 1975; Lafontan and Agid 1978). Alternatively, the hypothalamus has also been demonstrated to influence fat mobilization through neural pathways in mammals (Kumom *et al.* 1976; Bray and Nishizawa 1978), and specific hypothalamic lesions cause elevated plasma FFA levels in the duck, *Anas platyrhynchos* (Hawkes and George 1975).

Effects of starvation on plasma FFA levels in teleosts vary. In the trout, *Salmo gairdneri* plasma FFA levels increase significantly after 5 days of fasting but are not consistently maintained over a 70 day period (Bilinski and Gardner 1968). Plasma FFA in the eel, *Anguilla anguilla* are significantly higher after 145 days of starvation compared to 8 (Larsson and Lewander 1973) but decrease during starvation in the toadfish, *Opsanus tau* (Tashima and Cahill 1965). Alternatively in the pike, *Esox lucius* plasma FFA concentrations remain uncharged during fasts of 1 to 7 days (Ince and Thorpe 1975) or up to 3 months (Ince and Thorpe 1976).

The present study was undertaken to further examine effects of $E_2$ and T on plasma FFA in the goldfish and to determine whether any action to raise plasma FFA levels could be mediated through the hypothalamus. In addition, data from uninjected control fish in 3

experiments are compared, indicating plasma FFA levels in goldfish after various periods of starvation.

# MATERIALS AND METHODS

## I. General Procedures and Standard Maintenance Conditions

General procedures for maintenance, intraperitoneal (Ip) injection and blood sampling of goldfish and preparation of steroid suspensions were as reported in previous chapters. The experiments were conducted in 96 litre flow-through aquaria under conditions of 12 ± 1°C and 12 hours light/12 hours dark photoperiod.

## II. Experiments

### *Steroid Injection Experiments*

#### *Experiment 3.1*

Female goldfish weighing 31.8 g (S.D. = 9.4) were acclimated to the standard conditions for 15 days in September 1977 prior to commencement of hormone and control treatment. The fish were fed twice daily *ad libitum* with Ewos Salmon Grower Extra pellets, size 5P, (Ewos Aquaculture International) throughout the experiment until they were starved for 24 hours prior to blood sampling. One control group was uninjected and the other was injected with vehicle. Experimental groups were treated with oestrone ($E_1$), oestradiol-17β ($E_2$) or testosterone (T). The fish were given 5 injections on alternate days (total 9 days) and were sampled 2 days after the final injection. The concentration of the $E_1$, $E_2$ and T suspensions was 100 µg/ml and the hormones were injected at a dose of 1.0 µg/2g body weight.

#### *Experiment 3.2*

Goldfish of both sexes weighing 25.9 g (S.D. = 5.1), were

acclimated to the standard conditions and fed twice daily as above for 17 days in October 1978. The next day was designated Day 0 and the fish were fed only once, at 09:30, after which food was withheld for the duration of the experiment. On Day 1, 7 fish were sampled and sacrificed as initial controls and on Day 2, hormonal and control treatment of the remaining fish commenced. The concentration of the $E_2$ and T suspensions was 200 µg/ml and the hormones were injected at a dose of 2.0 µg/2g body weight. One control group was injected Ip with vehicle and the other control group was uninjected. Fish from each of the 4 groups were sampled and sacrificed on Day 3. The remaining fish were treated again as before on Days 4 and 6 and were sampled and sacrificed on Day 7.

*Hypothalamic Implant Experiments*

*Experiment 3.3*

Male goldfish weighing 29.2 g (S.D. = 4.2) were acclimated to the standard conditions and fed twice daily as above for 8 days in March 1978 after which food was withheld for the duration of the experiment. On the 16th day of starvation, the fish were treated as described below. The fish were sampled 2 and 6 days post-treatment after which they were sacrificed.

Pellets weighing approximately 19 µg, containing approximately 1.3 µg of T with the balance consisting of cocoa butter, were prepared according to the procedure described by Billard and Peter (1977) and were implanted in the nucleus lateral tuberis (NLT) region of the hypothalamus employing the stereotaxic procedures described by Peter (1970) as modified by Peter and Gill (1975). Coordinates for implantation of pellets containing T or control pellets (cocoa butter only) were: 0.5, M, D 3.2 (Peter and Gill 1975). One additional

control group was implanted Ip with similar pellets containing T in the same quantity as implanted in the brain; another group was a normal (unimplanted) control.

*Experiment 3.4*

Female goldfish weighing 29.9 g (S.D. = 6.7) were acclimated to the standard conditions for 19 days in May 1979 and fed twice daily. The following day was designated Day 0 and the fish were fed only once, at 09:30. The fish were treated, as described below, on Day 2, and were sampled and sacrificed on Day 3. Fish that showed signs of illness were discarded from the experiment. Cocoa butter pellets weighing approximately 19 µg containing approximately 2.5 µg of $E_2$ or T and control pellets were prepared and implanted in the NLT as described for Experiment 3.3. Two additional control groups were implanted Ip with similar pellets containing $E_2$ or T in the same quantity as implanted in the brain.

III. Free Fatty Acids (FFA) Assay

Plasma FFA were determined using an adaptation of the method of Noma *et al.* (1973) and solutions were prepared according to those authors. Extraction solvent, 1.35 ml, copper reagent, 0.5 ml, and plasma, 50 µl, along with glass beads (added to prevent the formation of a stable emulsion) were shaken with a vortex mixer for 2 minutes after which 1.0 ml of upper, organic, phase was transferred to a second tube containing 0.2 ml of 2-(thiozolylazo)-*p*-cresol solution.

IV. Statistical Analysis

Mean levels of plasma FFA in groups of uninjected control fish

starved for varying periods of time were compared by Duncan's New Multiple Range test after preliminary analysis of variance (Steel and Torrie 1960). Mean plasma FFA levels of different groups in Experiments 3.1 through 3.4 were compared using the student's *t* test.

## RESULTS

### I. Effects of Starvation in Uninjected Fish

Plasma FFA levels from uninjected (normal) control groups in Experiments 3.1 (1 day of starvation) and 3.3 (17 and 21 days of starvation) and the initial (1 day of starvation) and uninjected control fish (3 and 7 days of starvation) from Experiment 3.2 are presented in Table 5. Plasma FFA levels increased with length of starvation.

### II. Effects of Steroid Injection

*Experiment 3.1*

Fish injected with $E_2$ or T had higher plasma FFA levels than the uninjected control fish in Experiment 1 but there were no significant differences between plasma FFA levels in the vehicle-injected fish and any other group (Table 6).

*Experiment 3.2*

On Day 3, after 1 hormone injection, plasma FFA levels in the T-injected fish were significantly higher than in the other 3 groups, and also higher than the levels in the initial controls (Fig. 22). On Day 7, after 3 injections, the T-, $E_2$- and vehicle-injected fish had higher plasma FFA levels than both the uninjected fish and the initial controls. The $E_2$- and vehicle-injected fish on Day 7 had higher plasma FFA levels than the corresponding fish on Day 3. There were no differences in plasma FFA levels between sexes in any group.

TABLE 5. Plasma free fatty acids (FFA) in uninjected goldfish starved for varying lengths of time.

| Group | Source | Length of Fast (Days) | Sex | FFA (mM) | |
|---|---|---|---|---|---|
| a | Experiment 3.1 (Uninjected controls) | 1 | F | 0.058±0.016 | (6) |
| b | Experiment 3.2 (Initial controls) | 1 | M,F | 0.096±0.020 | (7) |
| c | Experiment 3.2 (Uninjected controls) | 3 | M,F | 0.143±0.008 | (5) |
| d | Experiment 3.2 (Uninjected controls) | 7 | M,F | 0.144±0.020 | (7) |
| e | Experiment 3.3 (Normal controls) | 17 | M | 0.245±0.027 | (10) |
| f | Experiment 3.3 | 21 | M | 0.270±0.030 | (10) |

NOTE: FFA levels are mean ± S.E.
Numbers of goldfish in parentheses.

Results of Duncan's New Multiple Range test at indicated levels of significance. Groups underlined by the same line are not different at the indicated level of significance.

at $p < 0.05$ a b c d e f

at $p < 0.01$ a b c d e f

TABLE 6. Effects of injection of oestrone ($E_1$), oestradiol-17β ($E_2$) or testosterone (T) on plasma FFA in female goldfish (Experiment 3.1).

| Treatment | Plasma FFA (mM) | |
|---|---|---|
| Uninjected Controls | 0.058±0.016 | (6) |
| Vehicle | 0.096±0.026 | (7) |
| $E_1$ | 0.079±0.025 | (7) |
| $E_2$ | 0.127±0.024 | (7)* |
| T | 0.159±0.033 | (7)* |

NOTE: FFA levels are mean ± S.E.

Numbers of goldfish in parentheses.

* Significantly different from Uninjected Controls $p < 0.05$.

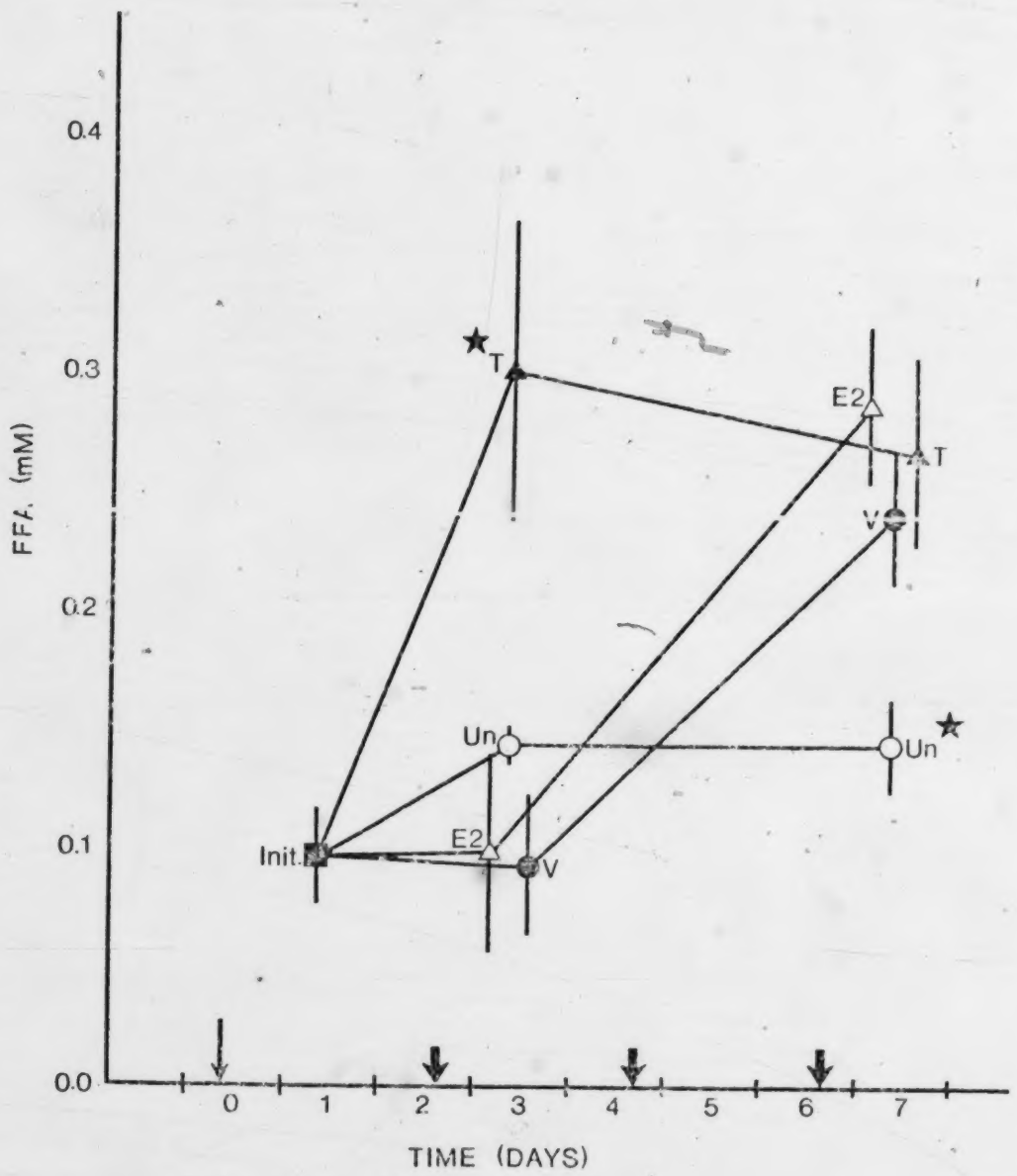
Figure 22. Plasma FFA concentrations (mean ± SE) in Initial Control fish (Init.), fish injected with testosterone (T), oestradiol-17β (E2) and Vehicle (V) and Uninjected control fish (Un).

On Day 3, T group (N=5) had higher plasma FFA levels than Un (N=5) and E2 (N=6), $p < 0.05$; V (N=6), $p < 0.02$ and Init. (N=7), $p < 0.01$.

On Day 7, Un group (N=7) had lower plasma FFA levels than T (N=7) or V (N=7), $p < 0.02$, and E2 (N=7), $p < 0.01$. E2 on Day 7 had higher plasma FFA than E2 on Day 3, $p < 0.01$ or Init., $p < 0.001$. V on Day 7 had higher plasma FFA than V on Day 3, $p < 0.01$ or Init., $p < 0.01$. T on Day 7 had higher plasma FFA than Init., $p < .01$.

= The time of last feeding, Day 0.

= The time of injections, Days 2, 4, and 6.


0.4
0.3
0.2
0.1
0.0
FFA. (mM)
T
E2
T
V
Un
Un
Init.
E2
V
0
1
2
3
4
5
6
7
TIME (DAYS)

III. Effects of Hypothalamic Implants

*Experiment 3.3*

There were no significant differences in plasma FFA levels between any 2 groups in the first sample (Table 7). In the second sample, fish implanted Ip with T had higher plasma FFA levels than the normal controls. Implantation of cocoa butter pellets containing T in the NLT had no effect on plasma FFA.

*Experiment 3.4*

Implantation of cocoa butter pellets containing $E_2$ or T in the NLT had no effect on plasma FFA (Table 8). There were no significant differences in plasma FFA levels between any of the groups.

TABLE 7. Plasma FFA after implantation of testosterone in the nucleus lateral tuberis (NLT) (Experiment 3.3).

| Treatment | Plasma FFA (mM) | |
|---|---|---|
| | Sample 1 | Sample 2 |
| Normal Control | 0.245±0.027 (10) | 0.270±0.030 (10) |
| Sham (Control NLT Implant) | 0.313±0.041 (10) | 0.346±0.034 (10) |
| T-NLT | 0.254±0.021 (13) | 0.294±0.026 (13) |
| T-Ip | 0.283±0.030 (10) | 0.353±0.025 (10)* |

NOTE: FFA levels are mean ± S.E.

Numbers of goldfish in parentheses.

T-NLT, T-Ip = T pellets implanted in the NLT or intraperitoneally, respectively.

* Significantly different from Normal Controls (Sample 2) $p < 0.05$.

TABLE 8. Plasma FFA after implantation of testosterone or oestradiol-17β in the NLT.

| Treatment | Plasma FFA (mM) | |
|---|---|---|
| $E_2$-NLT | 0.178±0.033 | (6) |
| T-NLT | 0.213±0.022 | (8) |
| $E_2$-Ip | 0.274±0.048 | (7) |
| T-Ip | 0.213±0.047 | (7) |
| Sham (Control NLT Implant) | 0.204±0.027 | (12) |

NOTE: FFA levels are mean ± S.E.

Numbers of goldfish in parentheses.

Abbreviations as in previous Tables.

There were no significant differences in plasma FFA between any groups.

## DISCUSSION

Plasma FFA levels were found to rise with increasing length of the fasting period in goldfish (Table 1). Increasing plasma FFA levels during fasting in mammals are associated with greater usage of FFA as an energy source (Newsholme and Start 1973). If the rate of utilization of plasma FFA in goldfish is proportional to the plasma levels, the present results suggest that lipid increases in importance as an energy source during fasting in the goldfish. This is consistent with the findings of Stimpson (1965) who reported depletion of liver lipid during fasting of goldfish maintained at 24°C, and the findings of Larsson and Lewander (1973) who reported depletion of liver and muscle TG concomitant with elevation of plasma FFA in *A. anguilla* during fasting.

In Experiment 3.2, where both sexes were used, there were no differences between sexes in plasma FFA levels in any one group. Whether or not sex or state of sexual maturity has any effect on plasma FFA in longer term fasting, reported here for males only, remains to be investigated. Sex distribution was not reported in the study of Tashima and Cahill (1965), and Larsson and Lewander (1973) worked with presumably immature fish ("yellow eels"). Bilinski and Gardner (1968) and Ince and Thorpe (1975, 1976) reported that both sexes were used in their studies but did not report any sex differences in FFA levels.

Plasma FFA levels in the $E_2$- and T-treated fish in Experiment 3.1 were higher than in the uninjected control group but not significantly different from the vehicle-injected controls. Although these results are suggestive of an effect of $E_2$ and T to raise plasma FFA, they are not definitive.

In Experiment 3.2, fish treated with T had significantly elevated concentrations of plasma FFA after 1 injection and 3 days of starvation but not after 3 injections and 7 days of starvation. This experiment failed to confirm the suggestion that injection of $E_2$ could raise plasma FFA levels in goldfish, or to extend that hypothesis to goldfish from the other vertebrates discussed above. The effect of T is particularly interesting in light of the observation that it has little effect on other plasma lipids in the dose used in the present study (Chapter 2), and induces vitellogenin synthesis in goldfish only after administration of massive doses (Hori *et al.* 1979).

Plasma androgen levels have been reported to increase with ovarian growth in the flounder, *Pseudopleuronectes americanus* (Campbell *et al.* 1976) and the plaice, *Pleuronectes platessa* (Wingfield and Grimm 1977) and also after injection of a mammalian gonadotrophin in female catfish, *Heteropneustes fossilis* (Truscott *et al.* 1978). However, the role of plasma androgens in female teleosts is not fully understood. The present results suggest that further study of metabolic effects of T, especially on plasma FFA, are warranted. The present findings are in contrast with those of Heald and Rookledge (1964) who reported decreased plasma FFA levels in domestic fowl after T injection.

The increased plasma FFA levels in the vehicle-injected controls on Day 7 of Experiment 3.2 is possibly a reflection of stress due to injection. This result contrasts with the data of Minick and Chavin (1972) who reported that sham or saline injection decreases plasma FFA levels in goldfish from 15 minutes to 12 hours post-injection. The plasma FFA response to vehicle injection in this study is over a

longer term than that reported by Minick and Chavin (1972).

Plasma FFA were higher in Experiment 3.4 on Day 3 than in the vehicle-injected fish on Day 3 in Experiment 3.2 ($p < .01$). This may be another manifestation of a stress response, evoked in this case by anaesthesia. All fish in Experiment 3.4 were anaesthetized for pellet implantation, either Ip or in the NLT, whereas in Experiment 3.2, fish were injected Ip on Day 2 without anaesthesia.

In Experiment 3.2, both the T-induced rise in plasma FFA on Day 3 and the increase in FFA in the vehicle-injected fish on Day 7 that was likely due to stress occurred after the fish had been starved for 3 days or more. On the other hand, there was no significant difference in plasma FFA levels between T-treated fish and vehicle-injected controls or between vehicle-injected controls and uninjected controls in Experiment 3.1 where the fish were fasted for only 24 hours and the levels were generally low compared to those in the starved fish. These observations suggest that responses of plasma FFA to various stimuli are dependent on the time of the last feeding.

The depression of plasma FFA in both control and hormone-treated fish fasted only 24 hours may be mediated through insulin which lowers plasma FFA levels in goldfish (Minick and Chavin, 1972) and *E. lucius* (Ince and Thorpe 1975). A single intra-arterial administration of glucose and arginine causes elevated plasma insulin levels which persist at near maximal levels for at least 6 hours in *A. anguilla* (Ince and Thorpe 1977). Insulin kinetics after feeding in the goldfish remain to be investigated. In prolonged fasting, insulin depression of plasma FFA would presumably be reduced or abolished.

In the present study, implantation of pellets containing $E_2$ or T in the hypothalamus had no effect on plasma FFA, and the results are not consistent with an action of sex steroids to raise plasma FFA being mediated through the hypothalamus. An alternative possibility is that sex steroids may have permissive actions on lipolysis that enhance the effects of other lipolytic agents; corticosteroids have such an effect in mammals (Goodman 1970). Attempts to elucidate lipolytic agents in teleosts have not proven fruitful; the *in vitro* studies of Farkas (1967, 1969) have indicated that hormones that are lipolytic in mammals antagonize lipolysis in teleosts.

# Chapter 4. PLASMA LIPOPROTEINS IN MALE GOLDFISH: A PRELIMINARY STUDY.

## INTRODUCTION

Because lipids are insoluble in physiological media, they are transported in animal plasma in association with proteins. The various lipid classes are usually distributed in different proportions on the several lipid transporting proteins in plasma, giving rise to lipoprotein classes of varying density. In the teleosts, lipid has been found to be associated with more than one serum or plasma lipoprotein in the sardine, *Sardinops caerula* (Lee and Puppione 1972), carp, *Cyprinus carpio* (Nakagawa 1979; Nakagawa *et al.* 1976), rainbow trout (Chapman *et al.* 1977; Perrier *et al.* 1979), coho salmon, *Oncorhynchus kisutch* (Vanstone and Ho 1961), and the flounder, *Platichthys flesus* (Emmersen and Petersen 1976). In addition to the normal circulating lipoproteins, the specific yolk precursor phospholipoprotein, vitellogenin (VG), is induced by oestrogen in female teleosts undergoing recrudescence (for review see this thesis, General Introduction). De Vlaming *et al.* (1977b) have reported a VG-like lipoprotein in goldfish. In order to more fully understand the relationships between plasma lipid levels and VG, it is necessary to study plasma lipoproteins during the sexual cycle and after hormonal treatment.

Recently, Perrier *et al.* (1979) described a micro-modification of the lipoprotein separation procedure of Bernstein and Scholnick (1973) for use in *Salmo gairdneri*. Very low density lipoproteins (VLDL) and then low density lipoproteins (LDL) are selectively precipitated, ultimately leaving high density lipoproteins (HDL) still in solution.

The present study was designed to examine the suitability of the system of Perrier *et al.* (1979) for the study of plasma lipoproteins in vehicle- and oestrogen-treated goldfish, and to determine the lipid composition of the various fractions separated. In addition, electrophoretic separation of lipoproteins from whole plasma and from supernatants remaining after precipitations was performed in order to determine whether material being precipitated could be qualitatively identified.

## MATERIALS AND METHODS

### I. General Procedures and Maintenance Conditions

General procedures for maintenance, intraperitoneal (Ip) injection, blood sampling, preparation of steroid suspensions and plasma lipid analyses were as reported in previous chapters. The experiment was conducted in 96 litre flow-through aquaria under conditions of 12 ± 1°C and 12 hours light/12 hours dark photoperiod.

### II. Experimental Protocol

Male goldfish weighing 29.9 g (S.D. = 6.7) were acclimated to the above conditions for 17 days in July 1979 prior to commencement of hormone or control injection. Throughout the experiment, the goldfish were fed twice daily with Ewos Salmon Grower Extra pellets, size 5P, except for a 24-hour fast prior to blood sampling. Hormone-treated fish were injected with a suspension containing 200 µg oestrone ($E_1$) and 200 µg oestradiol-17β ($E_2$)/ml. The injection dose was 4.0 µg total oestrogen/2g body weight. The fish were injected with hormone or control (vehicle) solution 7 times over a 14 day period. Blood was sampled the morning after the last injection.

### III. Lipoprotein Precipitation

Pools of haemolysis-free plasma were prepared from each treatment group and aliquots were set aside for lipid analysis and electrophoresis. Lipoprotein precipitation was performed according to the procedure described by Perrier *et al.* (1979). Plasma (1.5 ml) was

added to 0.04 ml of 1.8% dextran sulphate (DS - molecular weight 40,000) and 0.04 ml of 1M $MnCl_2$. The mixture was incubated for 80 minutes at room temperature and then centrifuged at 40,000 *g* for 15 minutes. After 0.7 ml of the supernatant (S1) was removed, a further 0.04 ml of DS and 0.04 ml $MnCl_2$ solutions were added to the remainder. The mixture was incubated for a further 20 minutes at room temperature, centrifuged at 40,000 *g* for 15 minutes and a portion of supernatant (S2) was removed. Whole plasma, S1 and S2 were assayed for triglycerides (TG), total cholesterol (TC) and lipid phosphorus (LP).

IV. Electrophoresis

Electrophoresis using 1 inch wide strips of Whatman No. 1 Filter paper was performed for 21 hours on 10 µl samples of whole plasma and supernatants at a current of 0.5 mA per strip. The buffer was 0.05 M sodium barbital in 20% methanol containing 0.1% bovine serum albumin and titrated to pH 8.6 with phosphoric acid. Lipoproteins were stained with saturated Oil Red O in 60% ethanol and mobilities compared to bromthymol blue.

## RESULTS

Concentrations of TG, TC and LP in the pooled plasma from oestrogen- and vehicle-injected fish appear in Table 9. The proportional lipid content of the precipitates, designated P1 and P2, formed after each centrifugation was calculated by difference (Perrier *et al.* 1979). The remaining lipid was in solution after the second centrifugation (S2).

Qualitative observations of the precipitates indicated that in both groups, little precipitation occurred after one addition of DS and $MnCl_2$ solutions, although a white substance appeared after addition of $MnCl_2$ to the plasma from oestrogen-treated fish. A heavy precipitation occurred after the second addition of DS and $MnCl_2$ solutions in the plasma from the vehicle-injected fish but not in that from the oestrogen-treated fish. After the second centrifugation, a white layer was present on the surface of the plasma from the oestrogen-treated fish but not on that from the vehicle-injected fish. The white material in plasma from oestrogen-treated fish is likely manganese oxide (Burnstein and Scholnick 1973).

Levels of TG and TC were similar in the two plasma pools but the LP concentration was much higher in the plasma pool from oestrogen-treated fish. A higher proportion of all three lipid classes was precipitated after two additions of DS and $MnCl_2$ in the vehicle-injected fish. This was especially true for TG.

Paper electrophoresis of whole plasma from vehicle-injected fish revealed two broad lipoprotein bands, one with a mobility relative to the tracer ($R_M$) of 0.0 - 0.26 (Band 1) and the second with $R_M$ of

TABLE 9. Plasma lipid concentrations and lipid distribution among plasma fractions in control and oestrogen-treated male goldfish.

| Fraction | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Vehicle | | | Oestrogen | | |
| | TG (mM) | TC (mg/dl) | LP (mg/dl) | TG (mM) | TC (mg/dl) | LP (mg/dl) |
| Whole Plasma | 6.00 | 239 | 28.5 | 5.91 | 262 | 56.2 |
| | Proportion of each of the above lipids in each fraction | | | | | |
| P1 | 19.6% * | 6.6% | 0% | 13.0% | 13.1% | 7.0% |
| P2 | 44.8% | 39.5% | 29.9% | 28.7% | 22.7% | 7.8% |
| Total Precipitate (P1 + P2) | 64.4% | 46.1% | 29.9% | 41.7% | 35.8% | 14.8% |
| S2 | 35.6% | 53.9% | 70.1% | 58.2% | 64.2% | 85.1% |

NOTE: P1 and P2, lipid removed from plasma in first and second precipitation, respectively.

S2 = lipid in S2 supernatant.

* For example, 19.6% of plasma TG from vehicle-injected fish was in P1.

0.34 - 0.54 (Band 2). In the S1 fraction, Band 1 was resolved into a distinct Band 1a, $R_M$ = 0.04 and a broad Band 1b, $R_M$ = 0.04 - 0.26. Band 2 remained unchanged. In the S2 fraction, Band 1a was sharply reduced and Band 1b was eliminated. Band 2 was resolved into 2 components, 2a, $R_M$ = 0.34 and 2b, $R_M$ = 0.41 - 0.53.

Paper electrophoresis of whole plasma from the oestrogen-treated fish revealed a similar lipoprotein distribution to whole plasma from vehicle-injected fish with the exception of a third band (Band 3) at $R_M$ = 0.30. Unlike the case with plasma from the vehicle-injected fish, resolution was not improved and no band was eliminated in the S1 and S2 fraction.

## DISCUSSION

The combination of the lipoprotein precipitation technique of Perrier *et al.* (1979) applied to goldfish plasma and electrophoresis as described above did not demonstrate separation of VLDL from LDL. The first precipitation, which removes VLDL from trout plasma (Perrier *et al.* 1979) did remove some lipid from plasma of control fish (Table 9) but no resolved lipoprotein band present in the whole plasma electropherogram was absent from the S1 electropherogram. Therefore, no definable fraction was removed by the first precipitation. It may be that the precipitation conditions developed for trout require modification for adaptation to goldfish material or that better electrophoretic resolution of lipoproteins, or a combination of both, is necessary to demonstrate VLDL separation from LDL. Burnstein and Scholnick (1973) also reported coprecipitation of VLDL and HDL with a DS-$MnCl_2$ system. Whether or not the molecular weight of DS used has an effect on precipitation characteristics is unclear. Neither Perrier *et al.* (1979) nor Burnstein and Scholnick (1973) reported the molecular weight of their DS preparation.

Despite the above difficulty, some pertinent information can be gleaned from this preliminary study. After two additions of DS and $MnCl_2$ solutions, a large precipitate was obtained from the control plasma which was likely VLDL plus LDL (Perrier *et al.* 1979). Electropherograms of the remaining supernatant, S2, from the present study revealed almost total elimination of the slow moving Band 1. In pilot studies on control plasma, Band 1 was absent in S2. The high proportion of total plasma phospholipids that remained in supernatant

S2 and the comparatively high electrophoretic mobility of these lipoproteins support the contention that S2 contains HDL (Lee and Puppione 1972; Perrier *et al.* 1979).

The other important finding was the difference in precipitation characteristics in plasma between control and oestrogen-treated fish in the DS-$MnCl_2$ system. Unlike control plasma two additions of DS and $MnCl_2$ solutions did not cause formation of a large visible precipitate from plasma of oestrogen-treated fish and no elimination of electrophoretic bands occurred. The reduction in lipid content in the supernatants after each addition of DS and $MnCl_2$ indicates that lipoproteins were precipitated but that selective precipitation of major classes did not occur. Therefore, despite the similar concentrations of plasma TG and TC in control and oestrogen-treated fish in this experiment, some characteristic of the lower density lipoproteins was altered. The nature of this alteration is unknown.

Electrophoresis of whole plasma from oestrogen-treated fish revealed a third band (Band 3) with $R_M$ between those of Bands 1 and 2. However, since resolution of Band 2 from control plasma reveals a band of similar $R_M$ to Band 3, it is unclear whether Band 3 is unique to oestrogen-treated fish.

Plasma concentrations of TC and TG were similar in the plasma pools from oestrogen-treated and control fish, suggesting that unlike the case with females, oestrogen had little effect on these lipids. However, the TG concentration in the control fish was similar to that of maturing females in which oestrogen injection had no effect and the TC concentration in the pool from oestrogen-treated fish was similar to those in maturing fish after oestrogen treatment (this thesis,

Chapter 2). Studies on plasma lipids in male goldfish during the sexual cycle are lacking.

The phospholipid concentration in the plasma pool from oestrogen-treated fish was approximately double that in the control pool. The extra phospholipid was contained entirely in the S2 (HDL) fraction (Table 9).

The present study has demonstrated that goldfish lipoproteins may be separated into at least two fractions but that modifications of the system used must be undertaken in order to allow separation of the three major classes of lipoproteins that have been found in some other teleosts. Oestrogen treatment of male goldfish had little influence on the plasma concentration of neutral lipids but the lighter lipoproteins, which consist largely of neutral lipids, were altered in a manner which remains to be investigated.

# GENERAL DISCUSSION

The foregoing chapters have provided evidence for the dependence of the plasma concentrations of three major classes of lipids on the reproductive state of the female goldfish, and descriptions of the effects of SG-G100 and sex steroids on plasma concentrations of these lipids. In addition, data concerning the relation of plasma FFA to duration of fasting period, effects of sex steroids on plasma FFA and distributions of plasma lipids on lipoproteins in the goldfish are presented. This section progresses from the more strict interpretation of experimental results presented in the chapters above to a consideration of the broader implications of these findings.

Plasma triglyceride (TG) concentrations were found to increase with increasing ovarian size in goldfish maintained under standardized conditions at 12°C for 2 weeks. This increase was attributed to the rising plasma oestrogen levels associated with progression of ovarian development. Injection of SG-G100 caused increased plasma TG concentrations in fish with small ovaries and this response was also attributed to oestrogen. The effect of injected oestrone ($E_1$) to increase plasma TG concentrations in sexually regressed fish supports the involvement of oestrogen in the raising of plasma TG levels, thus increasing the availability of that lipid to potential sites of utilization, such as the ovary. It was suggested in Chapter 1 that the increase in plasma TG with increasing GSI in fish held at 12°C can be arbitrarily divided at GSI = 6; plasma TG levels increased with increasing GSI in those fish with GSI less than 6 and plateaued above

that boundary. The TG releasing mechanisms may therefore be operating near maximal capacity in sexually maturing female goldfish that have reached a GSI intermediate between the regressed and mature states, which would explain why injection of oestrogen did not affect the plasma TG levels of sexually maturing fish (Experiment 2.2, Fig. 14).

The ultimate fate of plasma TG in the female goldfish is unknown, although the results of this and other studies (Urist and Schjeide 1961; Takashima *et al.* 1972) are consistent with a major role of TG in the provision of fatty acids for oxidation, especially by the ovary, as well as for storage directly in the ovary. Such a role would be in contrast to the mammalian situation where free fatty acids (FFA) instead of TG are the major plasma source of lipid energy (Newsholme and Start 1973). Furthermore, important differences between teleosts and mammals in FFA metabolism, in terms of response to catecholamines, have been demonstrated in studies on goldfish (Minick and Chavin 1973), pike, *Esox lucius* (Ince and Thorpe 1975) and carp, *Cyprinus carpio*, bream, *Abramus brama*, and pikeperch, *Lucioperca lucioperca* (Farkas 1969). An important role for plasma TG as an energy source for migrating salmon has been postulated by Patton *et al.* (1970, 1975).

In man, removal of TG from the plasma is concentration dependent and follows Michaelis-Menten kinetics (Reaven *et al.* 1965; Steiner and Murase 1975). If a similar situation exists in goldfish, higher plasma concentrations of TG would be accompanied by a higher rate of removal of TG from the plasma, and presumably higher rates of utilization. Studies on the kinetics and fate of plasma TG in goldfish are necessary to test this hypothesis.

Mature teleost ovaries may consist of approximately 10 - 40% (of dry weight) lipid (Lapin 1973; Lizenko *et al.* 1973; Medford and Mackay 1978) of which between 10% and 35% may be TG (Shatunovskiy 1971; Lapin 1973; Lizenko *et al.* 1973). Although there are few studies on lipogenesis in fish tissues, the liver has been found to be the major lipogenic organ in several species (Klutymans and Zandee 1974; Aster and Moon 1977; Lin *et al.* 1977) with comparatively little activity in the ovary (Klutymans and Zandee 1974). If a similar situation prevails in goldfish, it is probable that the developing oocyte takes up TG (and other lipids) from the plasma. Extensive studies on the chicken show that most egg yolk lipids and lipoproteins are derived from the plasma (Schjeide *et al.* 1963; Gornall *et al.* 1972; Hillyard *et al.* 1972; Gornall and Kuksis 1973).

Lipid accumulation has been described in ultrastructural studies of oogenesis in a number of species (for general review, see Norrevang 1968; for teleosts, see Droller and Roth 1966; Upadhyay 1977). Lipid droplets appear in developing oocytes prior to pinocytotic accumulation of vitellogenin. These droplets subsequently disappear, presumably by inclusion into other yolk materials. The lipid droplets in teleost oocytes have been found in association with various organelles which were thought to be involved with their synthesis. However, the present results (Chapter 1) tend to support the contention of Raven (1961) that such globules arise from material previously accumulated in the cytoplasm.

Plasma TC concentrations were found to decline with increasing ovarian size in goldfish held under standardized conditions at 12°C.

Injection of SG-G100 in sexually maturing fish with small ovaries resulted in elevated plasma TC levels as did oestrogen injection in both sexually regressed and maturing fish. The response to SG-G100 was, as with the case of plasma TG, attributed to oestrogen. This action of oestrogen would increase availability of cholesterol to the ovary for uptake.

Total cholesterol comprises varying portions of the ovarian lipids in teleosts: ca. 20% in the cisco, *Coregonus albula* (Lizenko *et al.* 1973), ca. 25% in the cod, *Gadus morhua callarias* (Shatunovskiy 1971) and ca. 35% in the flounder, *Platichthys flesus bogdanovi* (Lapin 1973). Eckstein (1970) demonstrated the synthesis of cholesterol from acetate in ovarian homogenates of *Tilapia aurea*. However, Klutymans and Zandee (1974) provided data that suggest that lipid synthesis in the ovary of *E. lucius*, is minimal compared to that in other tissues (eg. liver), and that the rate of synthesis of cholesterol and cholesteryl esters is very low compared to that for other lipids. The rate of cholesterol synthesis has also been reported to be low in the Atlantic striped bass, *Roccus saxtilis* (Blondin *et al.* 1966) and the carp, *Scardinius erythropthalmus* (Saxena and Zandee 1969). Thus, it seems likely that a major portion of the ovarian cholesterol comes from exogenous sources, via the plasma. As ovarian development proceeds, uptake of plasma cholesterol by the ovary may slightly exceed output from the source(s), resulting in declining plasma concentrations with progression of ovarian growth. Testing this hypothesis requires measurement of turnover of plasma TC in goldfish under a variety of sexual conditions and the demonstration of some factor capable of

stimulating cholesterol uptake by oocytes. Results presented in Chapter 1 suggest that gonadotrophin may be involved in such an uptake. It is also possible that thyroid hormones are involved with cholesterol uptake since they have been found to lower plasma TC in the trout, *Salmo gairdnerii irideus* (Takashima *et al.* 1972) and to synergise with gonadotrophin in vitellogenesis in the goldfish (Hurlburt 1977).

Phospholipids have been found to comprise between 25% and 60% of the total ovarian lipids in the species studied by Shatunovskiy (1971), Lapin (1973) and Lizenko *et al.* (1973). Because the liver has been shown to be the main lipogenic organ in teleosts (see above), it is likely that much of the ovarian phospholipid is taken up from the plasma. The action of injected oestrogen to raise plasma LP that was demonstrated in this study (Chapter 2), would make phospholipids available to the ovary for uptake. In contrast to *P. flesus* (Petersen and Emmersen 1977), there is only a small difference in pre-treatment plasma LP levels between sexually regressed and sexually mature goldfish at 12°C (Chapter 1) and no difference between pre-treatment LP levels in sexually regressed and sexually maturing goldfish at 12°C (Chapter 2). These findings suggest that phospholipids destined for ovarian uptake in maturing female goldfish do not accumulate in the plasma or that such accumulation is masked by a corresponding decrease in plasma concentrations of other species of phospholipids.

At present, a factor capable of stimulating uptake of phospholipid from the plasma by a teleost ovary has not been identified. However, gonadotrophic preparations have been demonstrated to cause uptake of yolk protein by the ovary of the flounder, *Pseudopleuronectes*

*americanus* (Campbell and Idler 1976) and *S. gairdneri* (Campbell 1978), and to lower plasma LP in *Xenopus laevis* (Follett and Redshaw 1968). In this study, SG-G100 had little effect on plasma LP in the goldfish, but was implicated in the uptake of TG and TC by the ovary.

While the effects of SG-G100 on plasma TG and TC are consistent with gonadotrophin exerting a role in uptake of lipid from the plasma by the ovary in a GSI-dependent manner, the mechanism by which lipid uptake would occur remains to be investigated. The existence of a mammalian-like, progesterone-stimulated system for removal of TG from the plasma has not been supported in goldfish (Chapter 2). It may be that stimulation of uptake of plasma lipid by the ovary is a steroid-independent action of gonadotrophin. Oestradiol is without effect, and progesterone inhibits, the incorporation of vitellogenin (VG) by growing *Xenopus* oocytes *in vitro* (Wallace and Ho 1972), suggesting that gonadotrophin-induced VG incorporation may be independent of steroids in that species (Wiley and Dumont 1978).

Results presented in Chapters 1 and 2 indicate that pre-treatment levels of plasma LP were consistently correlated with TC, and also with TG in all but one case. On the other hand, pre-treatment levels of TC and TG were correlated in only 3 of 8 experiments. This latter observation, coupled with the different patterns of plasma TG and TC concentrations as gonad development proceeds (Figs. 1 and 2) suggests that, despite similar influences of sex hormones on TG and TC, other aspects of the control of their plasma levels are different.

It was found that the metabolic effects of SG-G100 and oestrogens on plasma TG and TC that were demonstrated in female goldfish main-

tained at 12°C were abolished in post-ovulatory fish at 21°C, despite a 2.5-fold increase in hormone dose. Yamazaki (1965) suggested that yolk formation is impaired in goldfish maintained at temperatures over 20°C. Despite high plasma concentrations of gonadotrophin, earlier stages of ovarian recrudescence in goldfish are inhibited at temperatures of 20°C or higher, indicating that ovarian responsiveness to gonadotrophin may be dependent on temperature (Gillet *et al.* 1978). Impairment of metabolic responses to gonadotrophin and sex steroids could be a factor in the sensitivity of recrudescence to high temperature. In this study, not all hormonal effects observed at 12°C were abolished at 21°C; SG-G100 induced ovulation in some fish (Experiment 1.4) and both $E_1$ and $E_2$ stimulated increases in plasma LP levels (Experiment 2.3. Fig. 18). The mechanism by which warm temperature could impair some of the animal's responses to gonadotrophin and steroids remains to be investigated.

Studies on the relation of plasma FFA levels to the ovarian cycle in teleosts are lacking. Shatunovskiy (1971) and Lapin (1973) reported the seasonal fluctuations in FFA levels as a percentage of total plasma lipids in *G. morhua callarias* and *P. flesus bogdanovi* respectively, but did not report actual plasma concentrations. In preliminary experiments in this study, it was found that plasma FFA levels were very low or undetectable in goldfish fasted only 24 hours, as in the standard experimental protocols described in Chapters 1 and 2. In this study, length of the fasting period prior to blood sampling has been found to influence plasma FFA levels in goldfish and responses of plasma FFA levels to hormonal challenge were also sensitive to the

length of the fast. The possible intervention of insulin in this process was discussed in Chapter 3. In addition to fasting, other factors, including both hormones and metabolites, influence plasma FFA levels in teleosts (Farkas, 1969; Minick and Chavin 1972, 1973; Ince and Thorpe 1975). The multiplicity of influences on plasma FFA obviously complicate the study of this lipid.

The role of FFA, if any, in gonad recrudescence is unclear. In addition to a possible use directly by the ovary, mobilized plasma FFA could serve as precursors for other lipids. In teleosts, plasma FFA of dietary origin have been demonstrated to be precursors of both plasma TG and phospholipids (Robinson and Mead 1973; Kayama and Iijima 1976). The present results are consistent with action of androgens stimulating mobilization of FFA in fasted animals for use either as precursors for other lipids or direct uptake by the gonad. The importance of such a process in fed fish remains uncertain as does the mechanism by which such mobilization could occur. Attempts to identify lipolytic factors which could mediate mobilization of FFA in teleosts have proven unsuccessful (Farkas 1967, 1969).

There is little information concerning the relation of increase in concentration of plasma lipids after oestrogen treatment to the oestrogen-induced synthesis of the yolk precursor, VG, in the goldfish. Augmentation in the plasma of a light lipoprotein, consisting largely of TG, in addition to the appearance of heavier yolk proteins in oestrogen-treated chickens was discussed by Schjeide *et al.* (1963). Redshaw and Follett (1971) demonstrated that *Xenopus* serum VG consisted of a high proportion of phospholipid, but that oocyte yolk platelets

had a higher content of neutral lipid, indicating that additional lipid incorporation into yolk must occur from a non-VG source. In the elasmobranch, *Scyliorhinus canicula*, oestrogen mobilizes lipid into the plasma in excess of that associated with VG (Craik, 1978b).

One means of assessing the relation of plasma lipids to VG is the study of plasma or serum lipoproteins. Ultimately, the composition and endocrine effects on the various lipoproteins, including VG, can be determined throughout the sexual cycle. Lipid has been found to be associated with more than one serum or plasma lipoprotein in a number of teleosts, including goldfish (for review see Chapter 4), and enhancement of 2 plasma lipoproteins by oestrogen treatment has been reported in *S. gairdnerii irideus* (Takashima *et al.* 1972) and the ayu, *Plecoglossus altivelis* (Aida *et al.* 1973a). Bailey (1957) concluded that increases in plasma LP concentrations in the goldfish after oestradiol benzoate injection were independent of those of other parameters associated with yolk protein production, such as protein phosphorus, which is a commonly used index of VG (Wallace and Jared 1968; Emmersen and Petersen 1976; Craik 1978a). These observations suggest that the transport and hence mobilization of lipid in teleosts are distinct from transport and mobilization of VG. This hypothesis is supported by the observations that phospholipids comprise approximately 85% - 90% of the lipid content of goldfish VG (Hori *et al.* 1979) but a much lower proportion of teleost ovarian lipids (see above) indicating that, as in *Xenopus*, other plasma lipids must be sequestered by the teleost ovary. The work reported in Chapter 4 comprises a preliminary study of plasma lipoproteins in the goldfish.

Electrophoretic analysis of control goldfish plasma revealed the presence of two major lipoprotein classes which could be separated with a polyanion-divalent metal precipitation system. The phospholipid content of the high density lipoprotein fraction of the pool from the oestrogen-treated fish in Chapter 4 was higher than in that from the control pool. Some of the additional phospholipid is likely due to the presence of VG in the high density fraction. Goldfish VG contains phospholipids (Hori *et al.* 1979), and VG from other vertebrates is a high density lipoprotein (Schjeide *et al.* 1963). However, as stated above, Bailey (1957) found that oestrogen-induced increases in serum lipid phosphorus levels in goldfish were independent of VG. In addition, Craik (1978a) found sex differences in plasma lipid phosphorus levels in *S. canicula* that were independent of VG. It is apparent, therefore, that changes in VG may not account for the entire difference in HDL phospholipid levels between oestrogen-treated and control fish. Further research is obviously necessary to clarify the relationships between lipid and VG metabolism in teleosts.

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